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(54) Title: HUMAN SLIT POLYPEPTIDE ZSLIT3

(57) Abstract: Slit protein polypetides, polynucleotides encoding the polypeptides, and related compositions and methods are disclosed. The polypeptides are expressed in human neuronal and organ tissues. The polypeptides may be used within methods for detecting receptors that mediate neurite outgrowth, modulate organogenesis, cellular proliferation and/or differentiation, and immune response.

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DESCRIPTION

HUMAN SLIT POLYPEPTIDE ZSLIT3

BACKGROUND OF THE INVENTION

Neuronal cell outgrowths, known as processes, grow away from the cell body to form synaptic connections. Long, thin processes that carry information away from the cell body are called axons, and short, thicker processes that carry information to and from the cell body are called dendrites. Axons and dendrites are collectively referred to as neurites. Neurites are extended by means of growth cones, the growing tip of the neurite, which is highly motile and is ultimately responsible for increasing and extending the neuronal network in the body. The growth cones are able to navigate their way to their targets using environmental cues or signals, which encourage or discourage the growth cone from extending the neurite in a particular direction. Such cues and signals include older neurons and orienting glial fibers, chemicals such as nerve growth factor released by astrocytes and other attracting or repelling substances released by target cells. The membrane of the growth cone bears molecules such as nerve cell adhesion molecule (N-CAM) that are attracted or repelled by environmental cues and thus influence the direction and degree of neurite growth. The growth cone also engulfs molecules from the environment which are transported to the cell body and influence growth. A number of proteins from vertebrates and invertebrates have been identified as influencing the guidance of neurite growth, either through repulsion or chemoattraction. Among those molecules are netrins, EPH-related receptor tyrosine kinases and their ligands, vitronectin, thrombospondin, human neuronal attachment factor-1 (NAF-1), connectin, adhesion molecules such as cell adhesion molecule(s) (CAM(s)) and the semaphorins/collapsins (Neugebauer et al., Neuron 6:345-58, 1991; O'Shea et al., Neuron 7:231-7, 1991; Osterhout et al., Devel. Biol. 150:256-65, 1992;

Goodman, Cell 78:353-6, 1993; DeFreitas et al., Neuron 15:333-43, 1995; Dodd and Schuchardy Cell 81:471-4, 1995; Keynes and Cook, Cell 83:161-9, 1995; Müller et al., Cur. Opin. Genet. and Devel. 6:469-74, 1996, Goodman, Annu. Rev. Neurosci. 19:341-77, 1996; WIPO Patent Application No: 97/29189 and Goodman et al., US Patent No. 5,639,856).

Semaphorins/collapsins are a family of related transmembrane and secreted molecules. Invertebrate, vertebrate and viral semaphorins are known (Kolodkin et al., Cell 75:1389-99, 1993; Luo et al., Cell 75:217-27, 1993; Ensser and Fleckenstein, J. Gen. Virol. 76:1063-7, 1995; Luo et al, Neuron 14:1131-40, 1995; Adams et al., Mech. Devel. 57:33-45, 1996; Hall et al., Proc. Natl. Acad. Sci. USA 93:11780-8, 1996; Roche et al., Oncogene 12:1289-97, 1996; Skeido et al., Proc. Natl. Acad. Sci. USA 93:4120-5, 1996; Xiang et al., Genomics 32:39-48, 1996; Eckhardt et al., Mol. Cell Neurosci. 9:409-19, 1997 and Zhou et al., Mol. Cell. Neurosci. 9:26-41, 1997).

In addition, there is a family of secreted proteins related to *Drosophila* slit proteins that are required for normal neuronal development. Slit proteins found in *Drosophila*, humans, mice, and other species are involved in directing neuronal guidance. Slit proteins act primarily in repulsive axon guidance, such as chemorepulsion and collapsing activity, but may have other functions in neuronal growth and organogenesis, e.g., neuronal cell migration. Structurally, slit protein family members are characterized by leucine rich repeats (LRRs) and epidermal growth factor (EGF)-like repeats. However they may also contain Ig-like domains, as well as other structural features. Thus far, all slit proteins have been shown to be expressed in neuronal tissues and cells, including forebrain, hippocampus, cerebellar granule cells, glial cells, motor neurons, and spinal cord; however, some data show additional expression in other tissues such as endocrine tissues (e.g. thyroid).

Moreover, proliferation and differentiation of cells of multicellular organisms are controlled by hormones and polypeptide growth factors. These diffusible molecules allow cells to communicate with each other and act in concert to regulate cells and form organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the steroid hormones (e.g. estrogen, testosterone),

parathyroid hormone, follicle stimulating hormone, the interleukins, platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin (EPO) and calcitonin. Hormones and growth factors influence cellular metabolism by binding to proteins. These proteins may be integral membrane proteins that are linked to signaling pathways within the cell, such as second messenger systems. Other classes of proteins that hormones and growth factors influence are soluble molecules, such as the transcription factors. There is a continuing need to discover new hormones, growth factors and the like. The *in vivo* activities of these molecules illustrates the enormous clinical potential of, and need for, similar proteins, their agonists and antagonists.

Proteins affecting neurite growth cues are of great therapeutic value. Isolating and characterizing novel slit proteins would be of value for example, in modulating neurite growth and development; organ regeneration; treatment of peripheral neuropathies; for use as therapeutics for the regeneration of neurons following strokes, brain damage caused by head injuries and paralysis caused by spinal injuries; diagnosing neurological diseases and in treating neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease, and the like. In addition, slit proteins found in non-neuronal tissues and are useful for modulating cellular activation, homing, targeting, adhesion, proliferation and differentiation as well as mediating immunological responses. The present invention addresses these needs and others by providing novel slit proteins and related compositions and methods.

DESCRIPTION OF THE INVENTION

The present invention provides novel polynucleotides, polypeptides and related compositions and methods.

Within one aspect, the present invention provides an isolated polynucleotide that encodes a slit protein polypeptide comprising a sequence of amino acid residues that is at least 90% identical to an amino acid sequence selected from the group consisting of: (a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe); (b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid

number 673 (Ile); and (c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile). In one embodiment, the isolated polynucleotide disclosed above is selected from the group consisting of: (a) a polynucleotide sequence as shown in SEQ ID NO:1 from nucleotide 157 to nucleotide 861; (b) a polynucleotide sequence as shown in SEQ ID NO:1 from nucleotide 70 to nucleotide 2019; (c) a polynucleotide sequence as shown in SEQ ID NO:1 from nucleotide 1 to nucleotide 2019; and (d) a polynucleotide sequence complementary to (a), (b), or (c). In another embodiment, the isolated polynucleotide disclosed above comprises nucleotide 1 to nucleotide 2019 of SEQ ID NO:3. In another embodiment, the isolated polynucleotide disclosed above comprises a sequence of amino acid residues selected from the group consisting of: (a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe); (b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and (c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile).

Within a second aspect, the present invention provides an expression vector comprising the following operably linked elements: a transcription promoter; a DNA segment encoding a slit protein polypeptide as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and a transcription terminator, wherein the promoter is operably linked to the DNA segment, and the DNA segment is operably linked to the transcription terminator. In one embodiment the expression vector disclosed above further comprises a secretory signal sequence operably linked to the DNA segment.

Within a third aspect, the present invention provides a cultured cell comprising an expression vector as disclosed above, wherein the cell expresses a polypeptide encoded by the DNA segment.

Within a fourth aspect, the present invention provides a DNA construct encoding a fusion protein, the DNA construct comprising: a first DNA segment encoding a polypeptide comprising a sequence of amino acid residues selected from the group consisting of: (a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 23 (Gly); (b) the amino acid

sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 52 (Pro); (c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe); (d) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 288 (Pro), to amino acid number 408 (Asp); (e) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 298 (Asn), to amino acid number 350 (Pro); (f) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 409 (Cys), to amino acid number 441 (Cys); (g) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 442 (Glu), to amino acid number 673 (Ile); (h) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and at least one other DNA segment encoding an additional polypeptide, wherein the first and other DNA segments are connected in-frame; and wherein the first and other DNA segments encode the fusion protein.

Within another aspect, the present invention provides an expression vector comprising the following operably linked elements: a transcription promoter; a DNA construct encoding a fusion protein as disclosed above; and a transcription terminator, wherein the promoter is operably linked to the DNA construct, and the DNA construct is operably linked to the transcription terminator.

Within another aspect, the present invention provides a cultured cell comprising an expression vector as disclosed above, wherein the cell expresses a polypeptide encoded by the DNA construct.

Within another aspect, the present invention provides a method of producing a fusion protein comprising: culturing a cell as disclosed above; and isolating the polypeptide produced by the cell.

Within another aspect, the present invention provides an isolated slit protein polypeptide comprising a sequence of amino acid residues that is at least 90% identical to an amino acid sequence selected from the group consisting of: (a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe); (b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and (c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino

acid number 673 (Ile). In one embodiment, the isolated polypeptide disclosed above comprises a sequence of amino acid residues selected from the group consisting of: (a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe); (b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and (c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile).

Within another aspect, the present invention provides a method of producing a slit protein polypeptide comprising: culturing a cell as disclosed above; and isolating the slit protein polypeptide produced by the cell.

Within another aspect, the present invention provides a method of producing an antibody to a polypeptide comprising: inoculating an animal with a polypeptide selected from the group consisting of: (a) a polypeptide consisting of 30 to 649 amino acids, wherein the polypeptide is identical to a contiguous sequence of amino acids in SEQ ID NO:2 from amino acid number 24 (Cys) to amino acid number 673 (Ile); (b) a polypeptide as disclosed above; (c) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 52 (Pro); (d) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 288 (Pro), to amino acid number 408 (Asp); (e) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 298 (Asn), to amino acid number 350 (Pro); (f) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 409 (Cys), to amino acid number 441 (Cys); (g) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 442 (Glu), to amino acid number 673 (Ile); and wherein the polypeptide elicits an immune response in the animal to produce the antibody; and isolating the antibody from the animal.

Within another aspect, the present invention provides an antibody produced by the method as disclosed above, which binds to a polypeptide. In one embodiment, the antibody disclosed above is a monoclonal antibody. Within another aspect, the present invention provides an antibody that specifically binds to a polypeptide of SEQ ID NO:2.

Within another aspect, the present invention provides a method of detecting, in a test sample, the presence of a modulator of ZSLIT3 protein activity, comprising: transfecting a ZSLIT3-responsive cell, with a reporter gene construct that is responsive to a ZSLIT3-stimulated cellular pathway; and producing a ZSLIT3 polypeptide by the method as disclosed above; and adding the ZSLIT3 polypeptide to the cell, in the presence and absence of a test sample; and comparing levels of response to the ZSLIT3 polypeptide, in the presence and absence of the test sample, by a biological or biochemical assay; and determining from the comparison, the presence of the modulator of ZSLIT3 activity in the test sample.

Within another aspect, the present invention provides a method for detecting a genetic abnormality in a patient, comprising: obtaining a genetic sample from a patient; producing a first reaction product by incubating the genetic sample with a polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1, under conditions wherein said polynucleotide will hybridize to complementary polynucleotide sequence; visualizing the first reaction product; and comparing said first reaction product to a control reaction product from a wild type patient, wherein a difference between said first reaction product and said control reaction product is indicative of a genetic abnormality in the patient.

Within another aspect, the present invention provides a method for detecting a cancer in a patient, comprising: obtaining a tissue or biological sample from a patient; incubating the tissue or biological sample with an antibody as disclosed above under conditions wherein the antibody binds to its complementary polypeptide in the tissue or biological sample; visualizing the antibody bound in the tissue or biological sample; and comparing levels of antibody bound in the tissue or biological sample from the patient to a normal control tissue or biological sample, wherein an increase or decrease in the level of antibody bound to the patient tissue or biological sample relative to the normal control tissue or biological sample is indicative of a cancer in the patient.

Within another aspect, the present invention provides a method for detecting a cancer in a patient, comprising: obtaining a tissue or biological sample from a patient; labeling a polynucleotide comprising at least 14 contiguous nucleotides of

SEQ ID NO:1 or the complement of SEQ ID NO:1; incubating the tissue or biological sample with under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence; visualizing the labeled polynucleotide in the tissue or biological sample; and comparing the level of labeled polynucleotide hybridization in the tissue or biological sample from the patient to a normal control tissue or biological sample, wherein an increase or decrease in the labeled polynucleotide hybridization to the patient tissue or biological sample relative to the normal control tissue or biological sample is indicative of a cancer in the patient.

These and other aspects of the invention will become evident upon reference to the following detailed description.

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter:

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag. Affinity tags include a poly-histidine tract, protein A (Nilsson et al., EMBO J. 4:1075, 1985; Nilsson et al., Methods Enzymol. 198:3, 1991), glutathione S transferase (Smith and Johnson, Gene 67:31, 1988), Glu-Glu affinity tag (Grussenmeyer et al., Proc. Natl. Acad. Sci. USA 82:7952-4, 1995), substance P, Flag™ peptide (Hopp et al., Biotechnology 6:1204-10, 1988), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford et al., Protein Expression and Purification 2: 95-107, 1991. DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or

may encode polypeptides having altered amino acid sequence. The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

The terms "amino-terminal" and "carboxyl-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "complements of a polynucleotide molecule" is a polynucleotide molecule having a complementary base sequence and reverse orientation as compared to a reference sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a polynucleotide that has a contiguous stretch of identical or complementary sequence to another polynucleotide. Contiguous sequences are said to "overlap" a given stretch of polynucleotide sequence either in their entirety or along a partial stretch of the polynucleotide. For example, representative contigs to the polynucleotide sequence 5'-ATGGAGCTT-3' are 5'-AGCTTgagt-3' and 3'-tcgacTACC-5'.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide). Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

The term "expression vector" is used to denote a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may also include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, etc. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316:774-78, 1985).

An "isolated" polypeptide or protein is a polypeptide or protein that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The term "operably linked", when referring to DNA segments, indicates that the segments are arranged so that they function in concert for their intended purposes, e.g., transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α-globin, β-globin, and myoglobin are paralogs of each other.

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources,

synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

"Probes and/or primers" as used herein can be RNA or DNA. DNA can be either cDNA or genomic DNA. Polynucleotide probes and primers are single or double-stranded DNA or RNA, generally synthetic oligonucleotides, but may be generated from cloned cDNA or genomic sequences or its complements. Analytical probes will generally be at least 20 nucleotides in length, although somewhat shorter probes (14-17 nucleotides) can be used. PCR primers are at least 5 nucleotides in length, preferably 15 or more nt, more preferably 20-30 nt. Short polynucleotides can be used when a small region of the gene is targeted for analysis. For gross analysis of genes, a polynucleotide probe may comprise an entire exon or more. Probes can be labeled to provide a detectable signal, such as with an enzyme, biotin, a radionuclide, fluorophore, chemiluminescer, paramagnetic particle and the like, which are commercially available from many sources, such as Molecular Probes, Inc., Eugene, OR, and Amersham Corp., Arlington Heights, IL, using techniques that are well known in the art.

The term "promoter" is used herein for its art-recognized meaning to denote a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs

transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a protein encoded by a splice variant of an mRNA transcribed from a gene.

Molecular weights and lengths of polymers determined by imprecise analytical methods (e.g., gel electrophoresis) will be understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

All references cited herein are incorporated by reference in their entirety.

The present invention is based in part upon the discovery of a novel human member of the LRR-containing protein family, designated "ZSLIT3". The LRR-containing protein family is a diverse family of proteins with differing functions and cellular locations (Kobe, B. and Deisenhofer, J., Trends Biochem Sci. 19:415-21 1994). The ZSLIT3 polypeptide of the present invention shares the closest known structural similarity to *Drosophila* slit protein (Genbank Accession No. P24014). The human ZSLIT3 nucleotide sequence is represented in SEQ ID NO:1 and the deduced amino acid sequence in SEQ ID NO:2.

The novel human ZSLIT3 proteins and polypeptides encoded by polynucleotides of the present invention were initially identified by querying an Expressed Sequence Tag (EST) database for sequences homologous to conserved motifs within the slit protein family. Sequence analysis of the deduced amino acid sequence as represented in SEQ ID NO:2 indicates a 673 amino acid polypeptide containing a 23 amino acid residue secretory signal sequence (amino acid residues 1 (Met) to 23 (Gly) of SEQ ID NO:2), and a mature polypeptide of 650 amino acids (amino acid residues 24 (Cys) to 673 (Ile)). The mature ZSLIT3 polypeptide sequence contains the following domains, and motifs:

(1) an "N-terminal LRR flanking domain" comprising amino acid residues 24 (Cys) to 52 (Pro) of SEQ ID NO:2). Within the N-terminal LRR flanking domain are 4 conserved cysteines comprising amino acid residues 24, 28, 30 and 38 of SEQ ID NO:2.;

(2) a 235 amino acid residue "LRR domain" comprising amino acid residues 53 (Asp) to 287 (Phe) of SEQ ID NO:2). Within the LRR domain there are 10 consecutive LRR motifs, ordered from N-terminus to C-terminus:

"LRR-1" (corresponding to amino acids 53 (Asp) to 76 (Pro) of SEQ ID NO:2); "LRR-2" (corresponding to amino acids 77 (Gly) to 100 (Ala) of SEQ ID NO:2); "LRR-3" (corresponding to amino acids 101 (Asn) to 124 (Arg) of SEQ ID NO:2); "LRR-4" (corresponding to amino acids 125 (Arg) to 148 (Asp) of SEQ ID NO:2); "LRR-5" (corresponding to amino acids 149 (Arg) to 169 (Pro) of SEQ ID NO:2); "LRR-6" (corresponding to amino acids 170 (Arg) to 192 (Ala) of SEQ ID NO:2); "LRR-7" (corresponding to amino acids 193 (Asn) to 216 (Arg) of SEQ ID NO:2); "LRR-8" (corresponding to amino acids 217 (Asn) to 239 (Arg) of SEQ ID NO:2); "LRR-9" (corresponding to amino acids 240 (Gly) to 264 (Ala) of SEQ ID NO:2); and "LRR-10" (corresponding to amino acids 265 (Ala) to 287 (Phe) of SEQ ID NO:2);

(3) the LRR domain is followed by "middle region" of a stretch of amino acid residues comprising amino acid residues 288 (Pro) to 408 (Asp) of SEQ ID NO:2. Within this middle region is a conserved "C-terminal LRR flanking domain" to the LRR domain comprising amino acid residues 228 (Asn) to 350 (Pro) of SEQ ID NO:2. Within the C-terminal LRR flanking domain are 4 conserved cysteines comprising amino acid residues 302, 304, 328 and 349 of SEQ ID NO:2. The middle region is followed by

(4) an "EGF domain" comprising amino acid residues 409 (Cys) to 441 (Cys) of SEQ ID NO:2. Within the EGF domain are 6 conserved cysteines comprising amino acid residues 409, 414, 420, 430, 432 and 441 of SEQ ID NO:2. The EGF domain is followed by

(5) a "C-terminal region" comprising amino acid residues 442 (Glu) to 673 (Ile) of SEQ ID NO:2.

Moreover ZSLIT3 has putative N-linked glycosylation sites at Asn residues comprising amino acid residues 101, 117, 273, 500, and 528. Moreover, there are putative prenylation sites Cys residues comprising amino acid residues 304, 420 and 534.

Moreover the genomic structure of ZSLIT3 is readily determined by one of skill in the art by comparing the cDNA sequence of SEQ ID NO:1 and the translated amino acid of SEQ ID NO:2 with the genomic DNA in which the gene is contained (Genbank Accession No. AC012676). For example, such analysis can be readily done using FASTA as described herein. As such, the intron and exon junctions in this region of genomic DNA can be determined for the ZSLIT3 gene. This ZSLIT3 genomic DNA is located on chromosome 16, at 16q12 (Genbank Accession No. AC007226). Although the ZSLIT3 genomic DNA appears to have several exons, the entire coding sequence appears to be contained within in a single exon, as there are no putative introns within the genomic DNA. Thus, the present invention includes the ZSLIT3 gene as seen in human genomic DNA.

Those skilled in the art will recognize that domain boundaries are approximations based on sequence alignments, intron positions and splice sites, and may vary slightly; however, such estimates are generally accurate to within ± 4 amino acid residues.

The present invention is not limited to the expression of the sequence shown in SEQ ID NO:1. A number of truncated ZSLIT3 polynucleotides and polypeptides are provided by the present invention. These polypeptides can be produced by expressing polynucleotides encoding them in a variety of host cells. In many cases, the structure of the final polypeptide product will result from processing of the nascent polypeptide chain by the host cell, thus the final sequence of a ZSLIT3 polypeptide produced by a host cell will not always correspond to the full sequence encoded by the expressed polynucleotide. For example, expressing the complete ZSLIT3 sequence in a cultured mammalian cell is expected to result in removal of at least the secretory peptide, while the same polypeptide produced in a prokaryotic host would not be expected to be cleaved. By selecting particular combinations of polynucleotide and host cell, a variety of ZSLIT3 polypeptides can thus be produced. Differential processing of individual chains may result in heterogeneity of expressed polypeptides and the production of heterodimeric ZSLIT3 proteins. In addition, ZSLIT3 polypeptides can be produced by other known methods, such as solid phase synthesis, methods for which are well known in the art. See, for example, Merrifield, J.

Am. Chem. Soc. 85:2149, 1963; Stewart et al., Solid Phase Peptide Synthesis (2nd edition), Pierce Chemical Co., Rockford, IL, 1984; Bayer and Rapp, Chem. Pept. Prot. 3:3, 1986; and Atherton et al., Solid Phase Peptide Synthesis: A Practical Approach, IRL Press, Oxford, 1989.

Based on Northern data, ZSLIT3 polynucleotides are highly expressed in testis and ovary; expressed in brain; moderately expressed in heart, placenta, lung, liver, kidney, prostate and pancreas; and expressed at lower levels in brain, spleen, small intestine and colon and thyroid tissue, and other tissues (See, Example 2). The transcript size agrees with the predicted size of the ZSLIT3 protein as disclosed in SEQ ID NO:2. Additional analysis may reveal a ZSLIT3 transcript in numerous localized brain and neuronal tissues, or cells, and in tumor cell lines. RT-PCR data can also be performed to show where ZSLIT3 mRNA is expressed. Such methods are well known in the art and disclosed herein. Moreover, zslit3 is expressed in caners as evinced by expression in glioblastoma tissue, and intestinal carcinoma, osteogenic sarcoma and breast carcinoma cell lines, but not other tissues and cell lines tested (Example 3).

The present invention also provides polynucleotide molecules, including DNA and RNA molecules, that encode the ZSLIT3 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules. A degenerate polynucleotide sequence that encompasses all polynucleotides that encode the ZSLIT3 polypeptide of SEQ ID NO:2 (amino acid residues 1-673) is disclosed in SEQ ID NO:3. Thus, ZSLIT3 polypeptide-encoding polynucleotides ranging from nucleotide 1-2019 of SEQ ID NO:3 are contemplated by the present invention. Also contemplated by the present invention are fragments as described herein with respect to SEQ ID NO:1, which are formed from analogous regions of SEQ ID NO:3, wherein nucleotides 1-2019 of SEQ ID NO:1 correspond to nucleotides 1-2019 SEQ ID NO:3. Those skilled in the art will recognize that the degenerate sequence of SEQ ID NO:3 also provides all RNA sequences encoding SEQ ID NO:2 by substituting uracil (U) for thymine (T). The RNA equivalents of the herein named sequences are also contemplated by the present invention. Table 1 sets forth the one-letter nucleotide base codes used within SEQ ID NO:3 to denote degenerate nucleotide

positions. "Resolutions" are the nucleotides denoted by a nucleotide base code letter. "Complement" indicates the nucleotide base code for the complementary nucleotide(s). For example, the nucleotide base code "Y" denotes either the nucleotide C or T, and its complement nucleotide base code "R" denotes nucleotides A or G, A being complementary to T, and G being complementary to C.

TABLE 1

Nucleotide Base Code	Resolution	Nucleotide Base Code	Complement
A	A	T	T
C	C	G	G
G	G	C	C
T	T	A	A
R	A|G	Y	C|T
Y	C|T	R	A|G
M	A|C	K	G|T
K	G|T	M	A|C
S	C|G	S	C|G
W	A|T	W	A|T
H	A|C|T	D	A|G|T
B	C|G|T	V	A|C|G
V	A|C|G	B	C|G|T
D	A|G|T	H	A|C|T
N	A|C|G|T	N	A|C|G|T

The degenerate codons used in SEQ ID NO:3, encompassing all possible codons for a given amino acid, are set forth in Table 2.

TABLE 2

Three Letter Code	One Letter Code	Synonymous Codons	Degenerate Codon
Cys	C	TGC TGT	TGY
Ser	S	AGC AGT TCA TCC TCG TCT	WSN
Thr	T	ACA ACC ACG ACT	ACN
Pro	P	CCA CCC CCG CCT	CCN
Ala	A	GCA GCC GCG GCT	GCN
Gly	G	GGA GGC GGG GGT	GGN
Asn	N	AAC AAT	AAY
Asp	D	GAC GAT	GAY
Glu	E	GAA GAG	GAR
Gln	Q	CAA CAG	CAR
His	H	CAC CAT	CAY
Arg	R	AGA AGG CGA CGC CGG CGT	MGN
Lys	K	AAA AAG	AAR
Met	M	ATG	ATG
Ile	I	ATA ATC ATT	ATH
Leu	L	CTA CTC CTG CTT TTA TTG	YTN
Val	V	GTA GTC GTG GTT	GTN
Phe	F	TTC TTT	TTY
Tyr	Y	TAC TAT	TAY
Trp	W	TGG	TGG
Ter	.	TAA TAG TGA	TRR
Asn|Asp	B		RAY
Glu|Gln	Z		SAR
Any	X		NNN

One of ordinary skill in the art will appreciate that some ambiguity is introduced in determining a degenerate codon, representative of all possible codons encoding each amino acid. For example, the degenerate codon for serine (WSN) can, in some circumstances, encode arginine (AGR), and the degenerate codon for arginine (MGN) can, in some circumstances, encode serine (AGY). A similar relationship exists between codons encoding phenylalanine and leucine. Thus, some polynucleotides encompassed by the degenerate sequence may encode variant amino acid sequences, but one of ordinary skill in the art can easily identify such variant sequences by reference to the amino acid sequence of SEQ ID NO:2. Such variant sequences can be readily tested for functionality as disclosed herein.

One of ordinary skill in the art will also appreciate that different species can exhibit "preferential codon usage." In general, see, Grantham, et al., Nuc. Acids Res., 8:1893-912, 1980; Haas, et al. Curr. Biol., 6:315-24, 1996; Wain-Hobson, et al., Gene, 13:355-64, 1981; Grosjean and Fiers, Gene, 18:199-209, 1982; Holm, Nuc. Acids Res., 14:3075-87, 1986; Ikemura, J. Mol. Biol., 158:573-97, 1982. As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of all of the possible codons encoding each amino acid (See Table 2). For example, the amino acid threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species. Therefore, the degenerate codon sequence disclosed in SEQ ID NO:3 serves as a template for optimizing expression of polynucleotides in various cell types and species commonly used in the art and disclosed herein. Sequences containing preferential codons can be tested and optimized for expression in various species, and tested for functionality as disclosed herein.

The highly conserved amino acids in the LRR and EGF domains of ZSLIT3 can be used as a tool to identify new family members. For instance, reverse transcription-polymerase chain reaction (RT-PCR) can be used to amplify sequences, in particular, those sequences encoding the conserved LRR and EGF domains, especially sequences associated with the conserved cysteine residues, from RNA obtained from a variety of tissue sources or cell lines. In particular, highly degenerate primers designed from the ZSLIT3 nucleotide sequences as disclosed in SEQ ID NO:1 and SEQ ID NO:3 are useful for this purpose.

Within preferred embodiments of the invention, isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1, or to sequences complementary thereto, under stringent conditions. Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of SEQ ID NO:1, other polynucleotide probes, primers, fragments and sequences recited herein or sequences complementary thereto. Polynucleotide hybridization is well known in the art and widely used for many applications, see for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987; Berger and Kimmel, eds., Guide to Molecular Cloning Techniques, Methods in Enzymology, volume 152, 1987 and Wetmur, Crit. Rev. Biochem. Mol. Biol. 26:227-59, 1990. Polynucleotide hybridization exploits the ability of single stranded complementary sequences to form a double helix hybrid. Such hybrids include DNA-DNA, RNA-RNA and DNA-RNA.

Hybridization will occur between sequences which contain some degree of complementarity. Hybrids can tolerate mismatched base pairs in the double helix, but the stability of the hybrid is influenced by the degree of mismatch. The T_m of the mismatched hybrid decreases by 1°C for every 1-1.5% base pair mismatch. Varying the stringency of the hybridization conditions allows control over the degree of mismatch that will be present in the hybrid. The degree of stringency increases as the hybridization temperature increases and the ionic strength of the hybridization buffer decreases. Stringent hybridization conditions encompass temperatures of about 5-25°C below the thermal melting point (T_m) of the hybrid and a hybridization buffer having up

to 1 M Na^+. Higher degrees of stringency at lower temperatures can be achieved with the addition of formamide which reduces the T_m of the hybrid about 1°C for each 1% formamide in the buffer solution. Generally, such stringent conditions encompass temperatures of 20-70°C and a hybridization buffer containing up to 6X SSC and 0-50% formamide. A higher degree of stringency can be achieved at temperatures of from 40-70°C with a hybridization buffer having up to 4X SSC and from 0-50% formamide. Highly stringent conditions typically encompass temperatures of 42-70°C with a hybridization buffer having up to 1X SSC and 0-50% formamide. Different degrees of stringency can be used during hybridization and washing to achieve maximum specific binding to the target sequence. Typically, the washes following hybridization are performed at increasing degrees of stringency to remove non-hybridized polynucleotide probes from hybridized complexes.

The above conditions are meant to serve as a guide and it is well within the abilities of one skilled in the art to adapt these conditions for use with a particular polypeptide hybrid. The T_m for a specific target sequence is the temperature (under defined conditions) at which 50% of the target sequence will hybridize to a perfectly matched probe sequence. Those conditions which influence the T_m include, the size and base pair content of the polynucleotide probe, the ionic strength of the hybridization solution, and the presence of destabilizing agents in the hybridization solution. Numerous equations for calculating T_m are known in the art, see for example (Sambrook et al., ibid.; Ausubel et al., ibid.; Berger and Kimmel, ibid. and Wetmur, ibid.) and are specific for DNA, RNA and DNA-RNA hybrids and polynucleotide probe sequences of varying length. Sequence analysis software such as Oligo 4.0 (publicly available shareware) and Primer Premier (PREMIER Biosoft International, Palo Alto, CA) as well as sites on the Internet, are available tools for analyzing a given sequence and calculating T_m based on user defined criteria. Such programs can also analyze a given sequence under defined conditions and suggest suitable probe sequences. Typically, hybridization of longer polynucleotide sequences, >50 bp, is done at temperatures of about 20-25°C below the calculated T_m. For smaller probes, <50 bp, hybridization is typically carried out at the T_m or 5-10°C below. This allows for the maximum rate of hybridization for DNA-DNA and DNA-RNA hybrids.

The length of the polynucleotide sequence influences the rate and stability of hybrid formation. Smaller probe sequences, <50 bp, come to equilibrium with complementary sequences rapidly, but may form less stable hybrids. Incubation times of anywhere from minutes to hours can be used to achieve hybrid formation. Longer probe sequences come to equilibrium more slowly, but form more stable complexes even at lower temperatures. Incubations are allowed to proceed overnight or longer. Generally, incubations are carried out for a period equal to three times the calculated Cot time. Cot time, the time it takes for the polynucleotide sequences to reassociate, can be calculated for a particular sequence by methods known in the art.

The base pair composition of polynucleotide sequence will effect the thermal stability of the hybrid complex, thereby influencing the choice of hybridization temperature and the ionic strength of the hybridization buffer. A-T pairs are less stable than G-C pairs in aqueous solutions containing NaCl. Therefore, the higher the G-C content, the more stable the hybrid. Even distribution of G and C residues within the sequence also contribute positively to hybrid stability. Base pair composition can be manipulated to alter the T_m of a given sequence, for example, 5-methyldeoxycytidine can be substituted for deoxycytidine and 5-bromodeoxuridine can be substituted for thymidine to increase the T_m. 7-deazo-2'-deoxyguanosine can be substituted for guanosine to reduce dependence on T_m.

Ionic concentration of the hybridization buffer also effects the stability of the hybrid. Hybridization buffers generally contain blocking agents such as Denhardt's solution (Sigma Chemical Co., St. Louis, Mo.), denatured salmon sperm DNA, tRNA, milk powders (BLOTTO), heparin or SDS, and a Na^+ source, such as SSC (1X SSC: 0.15 M NaCl, 15 mM sodium citrate) or SSPE (1X SSPE: 1.8 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.7). By decreasing the ionic concentration of the buffer, the stability of the hybrid is increased. Typically, hybridization buffers contain from between 10 mM-1 M Na^+. Premixed hybridization solutions are also available from commercial sources such as Clontech Laboratories (Palo Alto, CA) and Promega Corporation (Madison, WI) for use according to manufacturer's instruction. Addition of destabilizing or denaturing agents such as formamide, tetralkylammonium salts, guanidinium cations or thiocyanate cations to the hybridization solution will alter the

T_m of a hybrid. Typically, formamide is used at a concentration of up to 50% to allow incubations to be carried out at more convenient and lower temperatures. Formamide also acts to reduce non-specific background when using RNA probes.

As previously noted, the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. In general, RNA is isolated from a tissue or cell that produces large amounts of ZSLIT3 RNA. Such tissues and cells are identified by Northern blotting (Thomas, Proc. Natl. Acad. Sci. USA 77:5201, 1980), and include breast, brain and neuronal tissues, although DNA can also be prepared using RNA from other tissues or isolated as genomic DNA. Total RNA can be prepared using guanidine isothiocyanate extraction followed by isolation by centrifugation in a CsCl gradient (Chirgwin et al., Biochemistry 18:52-94, 1979). Poly $(A)^+$ RNA is prepared from total RNA using the method of Aviv and Leder (Proc. Natl. Acad. Sci. USA 69:1408-12, 1972). Complementary DNA (cDNA) is prepared from poly$(A)^+$ RNA using known methods. Polynucleotides encoding ZSLIT3 polypeptides are then identified and isolated by, for example, hybridization or PCR.

A full-length clone encoding ZSLIT3 can be obtained by conventional cloning procedures. Complementary DNA (cDNA) clones are preferred, although for some applications (e.g., expression in transgenic animals) it may be preferable to use a genomic clone, or to modify a cDNA clone to include at least one genomic intron. Methods for preparing cDNA and genomic clones are well known and within the level of ordinary skill in the art, and include the use of the sequence disclosed herein, or parts thereof, for probing or priming a library. Expression libraries can be probed with antibodies to ZSLIT3, receptor fragments, or other specific binding partners.

The polynucleotides of the present invention can also be synthesized using techniques widely known in the art. See, for example, Glick and Pasternak, Molecular Biotechnology, Principles & Applications of Recombinant DNA, (ASM Press, Washington, D.C. 1994); Itakura et al., Annu. Rev. Biochem. 53: 323-56, 1984 and Climie et al., Proc. Natl. Acad. Sci. USA 87:633-7, 1990.

ZSLIT3 polynucleotide sequences disclosed herein can also be used as probes or primers to clone 5' non-coding regions of a ZSLIT3 gene. In view of tissue-

specific expression for ZSLIT3 elucidated by Northern blotting, this gene region is expected to provide for testis, ovary, brain, neurological, endrocrinological or tumor-specific expression. Promoter elements from a ZSLIT3 gene could thus be used to direct the tissue-specific expression of heterologous genes in, for example, transgenic animals or patients treated with gene therapy. Cloning of 5' flanking sequences also facilitates production of ZSLIT3 proteins by "gene activation" as disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous ZSLIT3 gene in a cell is altered by introducing into the ZSLIT3 locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a ZSLIT3 5' non-coding sequence that permits homologous recombination of the construct with the endogenous ZSLIT3 locus, whereby the sequences within the construct become operably linked with the endogenous ZSLIT3 coding sequence. In this way, an endogenous ZSLIT3 promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

The present invention further provides counterpart ligands and polynucleotides from other species (orthologs). These species include, but are not limited to, mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are ZSLIT3 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human ZSLIT3 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the ligand. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A ligand-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequence. A cDNA can also be cloned by PCR, using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the

cDNA of interest can be detected with an antibody to the ligand. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO:1 represents a single allele of the human ZSLIT3 gene and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO:2, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NO:2. cDNAs generated from alternatively spliced mRNAs, which retain the properties of the ZSLIT3 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

The present invention also provides isolated ZSLIT3 polypeptides that are substantially similar to the polypeptides of SEQ ID NO:2 and their orthologs. The term "substantially similar" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequences shown in SEQ ID NO:2 or their orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:2 or its orthologs). Percent sequence identity is determined by conventional methods. See, for example, Altschul et al., Bull. Math. Bio. 48: 603-16, 1986 and Henikoff and Henikoff, Proc. Natl. Acad. Sci. USA 89:10915-9, 1992. Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "blosum 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 3 (amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as:

$$\frac{\text{Total number of identical matches}}{\text{[length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences]}} \times 100$$

Table 3

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Sequence identity of polynucleotide molecules is determined by similar methods using a ratio as disclosed above.

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative variant ZSLIT3. The FASTA algorithm is described by Pearson and Lipman, Proc. Nat. Acad. Sci. USA 85:2444, 1988), and by Pearson, Meth. Enzymol. 183:63, 1990).

Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then re-scored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, J. Mol. Biol. 48:444, 1970; Sellers, SIAM J. Appl. Math. 26:787, 1974), which allows for amino acid insertions and deletions. Preferred parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62, with other perameters set as default. These parameters can be introduced into a FASTA program by modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, Meth. Enzymol. 183:63, 1990.

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons,

the ktup value can range between one to six, preferably from three to six, most preferably three, with other FASTA program parameters set as default.

The present invention includes nucleic acid molecules that encode a polypeptide having one or more conservative amino acid changes, compared with the amino acid sequence of SEQ ID NO:2. The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, Proc. Nat. Acad. Sci. USA 89:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. As used herein, the language "conservative amino acid substitution" refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. Preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3).

Variant ZSLIT3 polypeptides or substantially homologous ZSLIT3 polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 4) and other substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or an affinity tag. The present invention thus includes polypeptides of from about 253 amino acid residues to about 700 amino acid residues, that comprise a sequence that is at least 80%, preferably at least 90%, more preferably at least 95% or more identical to the corresponding region of SEQ ID NO:2, and more preferably having conserved cysteine residues corresponding to the amino acid residues 24, 28, 30, and 38; and/or 302, 304, 328, and 349; and/or 409,414, 420, 430, 432, and 441 of SEQ ID NO:2. Polypeptides comprising affinity tags can further comprise a

proteolytic cleavage site between the ZSLIT3 polypeptide and the affinity tag. Preferred such sites include thrombin cleavage sites and factor Xa cleavage sites.

Table 4

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, trans-3-methylproline, 2,4-methanoproline, cis-4-hydroxyproline, trans-4-hydroxyproline, N-methyl- glycine, allo-threonine, methylthreonine, hydroxy-ethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3 and 4-methylproline, 3,3-dimethyl-proline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluoro-phenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For

example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is carried out in a cell free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson et al., J. Am. Chem. Soc. 113:2722, 1991; Ellman et al., Methods Enzymol. 202:301, 1991; Chung et al., Science 259:806-9, 1993; and Chung et al., Proc. Natl. Acad. Sci. USA 90:10145-9, 1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti et al., J. Biol. Chem. 271:19991-8, 1996). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide et al., Biochem. 33:7470-6, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, Protein Sci. 2:395-3, 1993).

A limited number of non-conservative amino acids, amino acids that are not encoded by the generic code, non-naturally occurring amino acids, and unnatural amino acid residues may be substituted for ZSLIT3 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244: 1081-5, 1989; Bass et al., Proc. Natl. Acad. Sci. USA 88:4498-502, 1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., collapase activity, cellular interaction) to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-receptor interaction can also be determined by physical

analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction, or photoaffinity; in conjunction with mutation of putative contact site amino acids. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992. The identities of essential amino acids can also be inferred from analysis of homologies with related LRR and EGF polypeptides.

Determination of amino acid residues that are within regions or domains that are critical to maintaining structural integrity can be determined. Within these regions one can determine specific residues that will be more or less tolerant of change and maintain the overall tertiary structure of the molecule. Methods for analyzing sequence structure include, but are not limited to, alignment of multiple sequences with high amino acid or nucleotide identity and computer analysis using available software (e.g., the Insight II® viewer and homology modeling tools; MSI, San Diego, CA), secondary structure propensities, binary patterns, complementary packing and buried polar interactions (Barton, Current Opin. Struct. Biol. 5:372-376, 1995 and Cordes et al., Current Opin. Struct. Biol. 6:3-10, 1996). In general, when designing modifications to molecules or identifying specific fragments determination of structure will be accompanied by evaluating activity of modified molecules.

Amino acid sequence changes are made in ZSLIT3 polypeptides so as to minimize disruption of higher order structure essential to biological activity. For example, when the ZSLIT3 polypeptide comprises one or more helices, changes in amino acid residues will be made so as not to disrupt the helix geometry and other components of the molecule where changes in conformation abate some critical function, for example, binding of the molecule to its binding partners. The effects of amino acid sequence changes can be predicted by, for example, computer modeling as disclosed above or determined by analysis of crystal structure (see, e.g., Lapthorn et al., Nat. Struct. Biol. 2:266-268, 1995). Other techniques that are well known in the art compare folding of a variant protein to a standard molecule (e.g., the native protein). For example, comparison of the cysteine pattern in a variant and standard molecules can be made. Mass spectrometry and chemical modification using reduction and alkylation provide methods for determining cysteine residues which are associated with

disulfide bonds or are free of such associations (Bean et al., Anal. Biochem. 201:216-226, 1992; Gray, Protein Sci. 2:1732-1748, 1993; and Patterson et al., Anal. Chem. 66:3727-3732, 1994). It is generally believed that if a modified molecule does not have the same disulfide bonding pattern as the standard molecule folding would be affected. Another well known and accepted method for measuring folding is circular dichrosism (CD). Measuring and comparing the CD spectra generated by a modified molecule and standard molecule is routine (Johnson, Proteins 7:205-214, 1990). Crystallography is another well known method for analyzing folding and structure. Nuclear magnetic resonance (NMR), digestive peptide mapping and epitope mapping are also known methods for analyzing folding and structural similarities between proteins and polypeptides (Schaanan et al., Science 257:961-964, 1992).

A Hopp/Woods hydrophilicity profile of the ZSLIT3 protein sequence as shown in SEQ ID NO:2 can be generated (Hopp et al., Proc. Natl. Acad. Sci.78:3824-3828, 1981; Hopp, J. Immun. Meth. 88:1-18, 1986 and Triquier et al., Protein Engineering 11:153-169, 1998). The profile is based on a sliding six-residue window. Buried G, S, and T residues and exposed H, Y, and W residues were ignored. For example, in ZSLIT3, hydrophilic regions include: (1) amino acid number 123 (Leu) to amino acid number 128 (Arg) of SEQ ID NO:2; (2) amino acid number 156 (Gln) to amino acid number 161 (Arg) of SEQ ID NO:2; (3) amino acid number 225 (Asp) to amino acid number 230 (Arg) of SEQ ID NO:2; (4) amino acid number 322 (Ser) to amino acid number 327 (Arg) of SEQ ID NO:2; and (5) amino acid number 502 (Ser) to amino acid number 507 (Arg) of SEQ ID NO:2.

Those skilled in the art will recognize that hydrophilicity or hydrophobicity will be taken into account when designing modifications in the amino acid sequence of a ZSLIT3 polypeptide, so as not to disrupt the overall structural and biological profile. Of particular interest for replacement are hydrophobic residues selected from the group consisting of Val, Leu and Ile or the group consisting of Met, Gly, Ser, Ala, Tyr and Trp. For example, residues tolerant of substitution could include those hydrophobic residues as shown in SEQ ID NO: 2. Cysteine residues of SEQ ID NO: 2, will be relatively intolerant of substitution.

The identities of essential amino acids can also be inferred from analysis of sequence similarity between slit protein family members with ZSLIT3. Using methods such as "FASTA" analysis described previously, regions of high similarity are identified within a family of proteins and used to analyze amino acid sequence for conserved regions. An alternative approach to identifying a variant ZSLIT3 polynucleotide on the basis of structure is to determine whether a nucleic acid molecule encoding a potential variant ZSLIT3 polynucleotide can hybridize to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, as discussed above.

Other methods of identifying essential amino acids in the polypeptides of the present invention are procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244:1081 (1989), Bass et al., Proc. Natl Acad. Sci. USA 88:4498 (1991), Coombs and Corey, "Site-Directed Mutagenesis and Protein Engineering," in Proteins: Analysis and Design, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al., J. Biol. Chem. 271*:4699 (1996).

The present invention also includes functional fragments of ZSLIT3 polypeptides and nucleic acid molecules encoding such functional fragments. A "functional" ZSLIT3 or fragment thereof defined herein is characterized by its axon collapsing or slit protein-like activity, proliferative or differentiating activity, by its ability to induce or inhibit specialized cell functions, or by its ability to bind specifically to an anti-ZSLIT3antibody or ZSLIT3 receptor (either soluble or immobilized). As previously described herein, ZSLIT3 is characterized by a LRR domain structure and EGF domain, basic domain and other domains and motifs as described herein. Thus, the present invention further provides fusion proteins encompassing: (a) polypeptide molecules comprising one or more of the domains or motifs described above; and (b) functional fragments comprising one or more of these domains or motifs. The other polypeptide portion of the fusion protein may be contributed by another slit protein, or

by a non-native and/or an unrelated secretory signal peptide that facilitates secretion of the fusion protein.

Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a ZSLIT3 polypeptide. For example, DNA molecules having the nucleotide sequence of SEQ ID NO:1 or fragments thereof, can be digested with *Bal*31 nuclease to obtain a series of nested deletions. These DNA fragments are then inserted into expression vectors in proper reading frame, and the expressed polypeptides are isolated and tested for ZSLIT3 activity, or for the ability to bind anti-ZSLIT3 antibodies or ZSLIT3 receptor. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired ZSLIT3 fragment. Alternatively, particular fragments of a ZSLIT3 polynucleotide can be synthesized using the polymerase chain reaction.

Standard methods for identifying functional domains are well-known to those of skill in the art. For example, studies on the truncation at either or both termini of interferons have been summarized by Horisberger and Di Marco, Pharmac. Ther. 66:507 (1995). Moreover, standard techniques for functional analysis of proteins are described by, for example, Treuter et al., Molec. Gen. Genet. 240:113 (1993); Content et al., "Expression and preliminary deletion analysis of the 42 kDa 2-5A synthetase induced by human interferon," in Biological Interferon Systems, Proceedings of ISIR-TNO Meeting on Interferon Systems, Cantell (ed.), pages 65-72 (Nijhoff 1987); Herschman, "The EGF Receptor," in Control of Animal Cell Proliferation 1, Boynton et al., (eds.) pages 169-199 (Academic Press 1985); Coumailleau et al., J. Biol. Chem. 270:29270 (1995); Fukunaga et al., J. Biol. Chem. 270:25291 (1995); Yamaguchi et al., Biochem. Pharmacol. 50:1295 (1995); and Meisel et al., Plant Molec. Biol. 30:1 (1996).

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-7, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-6, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the

spectrum of allowable substitutions at each position. Other methods that can be used include phage display (e.g., Lowman et al., Biochem. 30:10832-7, 1991; Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204) and region-directed mutagenesis (Derbyshire et al., Gene 46:145, 1986; Ner et al., DNA 7:127, 1988).

Variants of the disclosed ZSLIT3 DNA and polypeptide sequences can be generated through DNA shuffling as disclosed by Stemmer, Nature 370:389-91, 1994 and Stemmer, Proc. Natl. Acad. Sci. USA 91:10747-51, 1994. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode active ligands or portions thereof (e.g., receptor-binding fragments) can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

Using the methods discussed herein, one of ordinary skill in the art can identify and/or prepare a variety of polypeptides that are substantially similar to amino acid residues 24 (Cys) to 673 (Ile), or a fragment thereof, allelic variants thereof and retain the properties of the wild-type ZSLIT3 protein. Such polypeptides may include additional amino acids from the N-terminal LRR flanking domain, LRR domain, LRR motifs LRR-1-10, middle region, C-terminal LRR flanking domain, EGF domain, C-terminal region and other domains and motifs as described herein; the secretory signal

sequence; affinity tags; and the like. Such polypeptides may also include additional polypeptide segments as generally disclosed herein.

The present invention further provides a variety of polypeptide fusions. For example, a ZSLIT3 polypeptide can be prepared as a fusion to a dimerizing protein as disclosed in U.S. Patents Nos. 5,155,027 and 5,567,584. Preferred dimerizing proteins in this regard include immunoglobulin constant region domains. Immunoglobulin-ZSLIT3 polypeptide fusions can be expressed in genetically engineered cells to produce a variety of multimeric ZSLIT3 analogs. Auxiliary domains can be fused to ZSLIT3 polypeptides to target them to specific cells, tissues, or macromolecules. For example, a ZSLIT3 polypeptide or protein could be targeted to a predetermined cell type by fusing a ZSLIT3 polypeptide to a ligand that specifically binds to a receptor on the surface of the target cell. In this way, polypeptides and proteins can be targeted for therapeutic or diagnostic purposes. A ZSLIT3 polypeptide can be fused to two or more moieties, such as an affinity tag for purification and a targeting domain. Polypeptide fusions can also comprise one or more cleavage sites, particularly between domains. See, Tuan et al., Connective Tissue Research 34:1-9, 1996.

For any ZSLIT3 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above.

The ZSLIT3 polypeptides of the present invention, including full-length polypeptides, fragments (e.g., receptor-binding fragments, growth cone directing fragments, immune response provoking fragments), and fusion polypeptides, can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor,

NY, 1989; and Ausubel et al., eds., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY, 1987.

In general, a DNA sequence encoding a ZSLIT3 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome. Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a ZSLIT3 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the ZSLIT3 polypeptide (amino acid residues 1 (Met) through amino acid residue 23 (Gly) of SEQ ID NO:2), or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is joined to the ZSLIT3 DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Alternatively, the secretory signal sequence contained in the polypeptides of the present invention is used to direct other polypeptides into the secretory pathway. The present invention provides for such fusion polypeptides. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from amino acid 1 (Met) to amino acid 23 (Gly) of SEQ ID NO:2 is operably linked to another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional

peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein. Such fusions may be used *in vivo* or *in vitro* to direct peptides through the secretory pathway.

Moreover, using methods described in the art, polypeptide fusions, or hybrid ZSLIT3 proteins, are constructed using regions or domains of the inventive ZSLIT3 in combination with those of other Slit protein family proteins (e.g. human SLIT-1 and SLIT-2, or murine slit proteins, and the like), or heterologous proteins (Sambrook et al., ibid.; Altschul et al., ibid.; Picard, Cur. Opin. Biology, 5:511-5, 1994, and references therein). These methods allow the determination of the biological importance of larger domains or regions in a polypeptide of interest. Such hybrids may alter reaction kinetics, binding, constrict or expand the substrate specificity, alter activity in neurite or other functional assays, alter immune response, or gene transcription in a cell, alter cytoskeletal organization and cell motility, transformation, or invasiveness, or alter tissue and cellular localization of a polypeptide, and can be applied to polypeptides of unknown structure.

Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding various components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. For example, part or all of a domain(s) conferring a structural or biological function may be swapped between ZSLIT3 of the present invention with the functionally equivalent domain(s) from another family member. Such domains include, but are not limited to the signal peptide, N-terminal LRR flanking domain, LRR domain, LRR motifs LRR 1-10, middle region, C-terminal LRR flanking domain, an EGF domain, C-terminal region and other domains and motifs as described herein. Such fusion proteins would be expected to have a biological functional profile that is the same or similar to polypeptides of the present invention or other known slit protein family proteins (e.g. affecting neurite growth or collapsing activity, and the like) depending on the fusion constructed. Moreover, such fusion proteins may exhibit other properties as disclosed herein.

Standard molecular biological and cloning techniques can be used to swap the equivalent domains between the ZSLIT3 polypeptide and those polypeptides to which they are fused. Generally, a DNA segment that encodes a domain of interest, e.g., a ZSLIT3 active polypeptide or motif described herein, is operably linked in frame to at least one other DNA segment encoding an additional polypeptide and inserted into an appropriate expression vector, as described herein. Generally DNA constructs are made such that the several DNA segments that encode the corresponding regions of a polypeptide are operably linked in frame to make a single construct that encodes the entire fusion protein, or a functional portion thereof. For example, a DNA construct would encode from N-terminus to C-terminus a fusion protein comprising a signal polypeptide followed by a mature polypeptide; or a DNA construct would encode from N-terminus to C-terminus a fusion protein comprising an N-terminal LRR flanking domain, followed by an LRR domain containing at least one LRR motif, followed by a C-terminal LRR flanking domain containing at least one EGF domain; or a DNA construct would encode from N-terminus to C-terminus a fusion protein comprising an LRR domain followed by an EGF domain ; or a DNA construct would encode from N-terminus to C-terminus a fusion protein comprising a signal peptide, N-terminal LRR flanking domain, LRR domain containing at least one LRR motif, middle region comprising a C-terminal LRR flanking domain, EGF domain, C-terminal region; or for example, any of the above as interchanged with equivalent regions from another slit protein family member. Such fusion proteins can be expressed, isolated, and assayed for activity as described herein. Moreover, such fusion proteins can be used to express and secrete fragments of the ZSLIT3 polypeptide, to be used, for example to inoculate an animal to generate anti-ZSLIT3 antibodies as described herein. For example a secretory signal sequence can be operably linked to the N-terminal LRR flanking domain, LRR domain containing at least one LRR motif, middle region comprising a C-terminal LRR flanking domain, EGF domain, C-terminal region, or a combination thereof (e.g., operably linked polypeptides comprising operably fused LRR domain and middle region comprising a C-terminal LRR flanking domain, with or without an EGF domain, or other ZSLIT3 polypeptide fragments or combinations described herein), to secrete a fragment of ZSLIT3 polypeptide that can be purified as described herein and

serve as an antigen to be inoculated into an animal to produce anti-ZSLIT3 antibodies, as described herein.

In addition, the proteins of the present invention (or polypeptide fragments thereof) can be joined to other bioactive molecules, particularly other slit proteins, to provide multi-functional molecules. For example, one or more domains from ZSLIT3 can be joined to other slit proteins to enhance their biological properties or efficiency of production.

The present invention thus provides a series of novel, hybrid molecules in which a segment comprising one or more of the domains of ZSLIT3 is fused to another polypeptide. Fusion is preferably done by splicing at the DNA level, as described herein, to allow expression of chimeric molecules in recombinant production systems. The resultant molecules are then assayed for such properties as enhanced or diminished neurite collapsing or repulsing activity, neuronal migration, increased or decreased immune response activity, improved solubility, improved stability, prolonged clearance half-life, improved expression and secretion levels, and pharmacodynamics. Such hybrid molecules may further comprise additional amino acid residues (e.g. a polypeptide linker) between the component proteins or polypeptides.

Cultured mammalian cells are suitable hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection (Wigler et al., Cell 14:725, 1978; Corsaro and Pearson, Somatic Cell Genetics 7:603, 1981; Graham and Van der Eb, Virology 52:456, 1973), electroporation (Neumann et al., EMBO J. 1:841-5, 1982), DEAE-dextran mediated transfection (Ausubel et al., ibid.), and liposome-mediated transfection (Hawley-Nelson et al., Focus 15:73, 1993; Ciccarone et al., Focus 15:80, 1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293 (ATCC No. CRL 1573; Graham et al., J. Gen. Virol. 36:59-72, 1977), Chinese hamster ovary (e.g., CHO-K1; ATCC No. CCL 61) cell lines

and DG44 CHO cells (Chasin et al., Som. Cell. Molec. Genet. 12:555-66, 1986). Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, e.g., U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978) and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (e.g., hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

In a preferred embodiment ZSLIT3 DNA fragments are subcloned into mammalian expression plasmids, such as pZP9 (ATCC No. 98668) or modifications thereof. For expression of affinity tagged ZSLIT3 proteins, Glu-Glu-tagged for example, such expression plasmids contain the mouse metallothionein-1 promoter; a TPA leader peptide followed by the sequence encoding a Glu-Glu tag (e.g., SEQ ID NO:4), for expression of N-terminal Glu-Glu ZSLIT3 proteins; the ZSLIT3 polynucleotide sequence without the native signal sequence, and a human growth hormone terminator. For expression of C-terminal Glu-Glu tagged proteins the expression cassette can be modified to place the sequence encoding a Glu-Glu tag (e.g., SEQ ID NO:4) after the ZSLIT3 nucleotide sequence followed by a stop codon and the

human growth hormone terminator. Within one preferred embodiment, such expression vectors would be transfected and expressed in mammalian cells, such as BHK or CHO cells. Transformed cells can be screened for expression of ZSLIT3 proteins by filter assay. Affinity tagged proteins can be detected using conjugated antibodies to the tag, such as anti-Glu-Glu antibody-HRP conjugate. Colonies expressing ZSLIT3 can be selected and subjected to Western Blot analysis and mycoplasma testing. Preferably individual clones can be expanded and used for large scale production of ZSLIT3 proteins.

Other higher eukaryotic cells can also be used as hosts, including plant cells, insect cells and avian cells. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar et al., J. Biosci. (Bangalore) 11:47-58, 1987. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino et al., U.S. Patent No. 5,162,222 and WIPO publication WO 94/06463. Insect cells can be infected with recombinant baculovirus, commonly derived from *Autographa californica nuclear polyhedrosis virus* (AcNPV). See, King, L.A. and Possee, R.D., The Baculovirus Expression System: A Laboratory Guide, London, Chapman & Hall; O'Reilly, D.R. et al., Baculovirus Expression Vectors: A Laboratory Manual, New York, Oxford University Press., 1994; and, Richardson, C. D., Ed., Baculovirus Expression Protocols. Methods in Molecular Biology, Totowa, NJ, Humana Press, 1995. The second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, V.A, et al., J Virol 67:4566-79, 1993). This system is sold in the Bac-to-Bac™ kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, pFastBac1™ (Life Technologies) containing a Tn7 transposon to move the DNA encoding the ZSLIT3 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." The pFastBac1™ transfer vector utilizes the AcNPV polyhedrin promoter to drive the expression of the gene of interest, in this case ZSLIT3. However, pFastBac1™ can be modified to a considerable degree. The polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as P*cor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins. See, Hill-Perkins, M.S. and

Possee, R.D., J. Gen. Virol. 71:971-6, 1990; Bonning, B.C. et al., J. Gen. Virol. 75:1551-6, 1994; and, Chazenbalk, G.D., and Rapoport, B., J. Biol. Chem. 270:1543-9, 1995. In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native ZSLIT3 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen, Carlsbad, CA), or baculovirus gp67 (PharMingen, San Diego, CA) can be used in constructs to replace the native ZSLIT3 secretory signal sequence. In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed ZSLIT3 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer, T. et al., Proc. Natl. Acad. Sci. 82:7952-4, 1985). Using a technique known in the art, a transfer vector containing ZSLIT3 is transformed into *E. coli*, and screened for bacmids which contain an interrupted lacZ gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is isolated, using common techniques, and used to transfect *Spodoptera frugiperda* cells, e.g. Sf9 cells. Recombinant virus that expresses ZSLIT3 is subsequently produced. Recombinant viral stocks are made by methods commonly used the art.

The recombinant virus is used to infect host cells, typically a cell line derived from the fall armyworm, *Spodoptera frugiperda*. See, in general, Glick and Pasternak, Molecular Biotechnology: Principles and Applications of Recombinant DNA, ASM Press, Washington, D.C., 1994. Another suitable cell line is the High FiveO™ cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent No.5,300,435). Commercially available serum-free media are used to grow and maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cellO405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. The cells are grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells at which time a recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3. Procedures used are generally described in available laboratory manuals (King, L. A. and Possee, R.D., ibid.; O'Reilly, D.R. et al., ibid.;

Richardson, C. D., ibid.). Subsequent purification of the ZSLIT3 polypeptide from the supernatant can be achieved using methods described herein.

Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing fragments or polypeptide fusions. Methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the POT1 vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., J. Gen. Microbiol. 132:3459-65, 1986; and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight et al., U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino et al., U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

The use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed in WIPO Publication WO 9717450. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the

plasmid be that of a *P. methanolica* gene. A preferred promoter is that of a *P. methanolica* alcohol utilization gene (*AUG1*). *P. methanolica* contains a second alcohol utilization gene, *AUG2*, the promoter of which can also be used. Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. This is conveniently accomplished by including 3' untranslated DNA sequence at the downstream end of the expression segment and relying on the promoter sequence at the 5' end. When using linear DNA, the expression segment will be flanked by cleavage sites to allow for linearization of the molecule and separation of the expression segment from other sequences (e.g., a bacterial origin of replication and selectable marker). Preferred such cleavage sites are those that are recognized by restriction endonucleases that cut infrequently within a DNA sequence, such as those that recognize 8-base target sequences (e.g., Not I). A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21). The *ADE2* gene, when transformed into an *ade2* host cell, allows the cell to grow in the absence of adenine. Other nutritional markers that can be used include the *P. methanolica ADE1*, *HIS3*, and *LEU2* genes, which allow for selection in the absence of adenine, histidine, and leucine, respectively. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Gene-deficient mutants can be prepared by known methods, such as site-directed mutagenesis. *P. methanolica* genes can be cloned on the basis of homology with their counterpart *Saccharomyces cerevisiae* genes.

Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. See, in general, Neumann et al., EMBO J. 1:841-5, 1982 and Meilhoc et al., Bio/Technology 8:223-7, 1990. For transformation of *P. methanolica*, electroporation is most efficient

when the cells are exposed to an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Prokaryotic host cells, including strains of the bacteria *Escherichia coli*, *Bacillus* and other genera are also useful host cells within the present invention. Techniques for transforming these hosts and expressing foreign DNA sequences cloned therein are well known in the art (see, e.g., Sambrook et al., ibid.). When expressing a ZSLIT3 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell. *P. methanolica* cells are cultured in a medium comprising adequate sources of carbon, nitrogen and trace nutrients at a temperature of about 25°C to 35°C. Liquid cultures are provided with sufficient aeration by conventional means,

such as shaking of small flasks or sparging of fermentors. A preferred culture medium for *P. methanolica* is YEPD.

Expressed recombinant ZSLIT3 polypeptides (or chimeric or fused ZSLIT3 polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, Affinity Chromatography: Principles & Methods, Pharmacia LKB Biotechnology, Uppsala, Sweden, 1988.

The polypeptides of the present invention can be isolated by exploitation of their physical or biochemical properties. Methods used to purify mammalian slit proteins, and LRR-containing proteins are exemplary (see, for example, Hu, H., Neuron 23:703-711, 1999). Moreover, immobilized metal ion adsorption (IMAC)

chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, Trends in Biochem. 3:1-7, 1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (Methods in Enzymol., Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, 1990, pp.529-39). Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (e.g., maltose-binding protein, Glu-Glu tag, or an immunoglobulin domain) may be constructed to facilitate purification.

It is preferred to purify the protein to >80% purity, more preferably to >90% purity, even more preferably >95%, and particularly preferred is a pharmaceutically pure state, that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. Preferably, a purified protein is substantially free of other proteins, particularly other proteins of animal origin.

ZSLIT3 polypeptides or fragments thereof may also be prepared through chemical synthesis. ZSLIT3 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; pegylated or non-pegylated; and may or may not include an initial methionine amino acid residue.

Polypeptides of the present invention can also be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. Methods for synthesizing polypeptides are well known in the art. See, for example, Merrifield, J. Am. Chem. Soc. 85:2149, 1963; Kaiser et al., Anal. Biochem. 34:595, 1970. After the entire synthesis of the desired peptide on a solid support, the peptide-resin is with a reagent which cleaves the polypeptide from the resin and removes most of the side-chain protecting groups. Such methods are well established in the art.

Polypeptides containing the receptor-binding region of the ligand can be used for purification of receptor. The ligand polypeptide is immobilized on a solid support, such as beads of agarose, cross-linked agarose, glass, cellulosic resins, silica-based resins, polystyrene, cross-linked polyacrylamide, or like materials that are stable under the conditions of use. Methods for linking polypeptides to solid supports are known in the art, and include amine chemistry, cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, and hydrazide activation. The resulting media will generally be configured in the form of a column, and fluids containing receptors are passed through the column one or more times to allow receptor to bind to the ligand polypeptide. The receptor is then eluted using changes in salt concentration, chaotropic agents ($MnCl_2$), or pH to disrupt ligand-receptor binding.

ZSLIT3 polypeptides or ZSLIT3 fusion proteins are used, for example, to identify the ZSLIT3 receptor. Using labeled ZSLIT3 polypeptides, cells expressing the receptor are identified by fluorescence immunocytometry or immunohistochemistry. ZSLIT3 polypeptides are useful in determining the distribution of the receptor on tissues or specific cell lineages, and to provide insight into receptor/ligand biology. An exemplary method to identify a ZSLIT3 receptor *in vivo* or *in vitro*, e.g., in cell lines, is to us a ZSLIT3 polypeptide fused to the catalytic domain of Alkaline phosphatase (AP), as described in Feiner, L. et al., Neuron 19:539-545, 1997. Such AP fusions, as well as radiolabeled ZSLIT3, ZSLIT3 fusions with fluorescent labels, and others described herein, combined with standard cloning techniques enable one of skill in the art to visualize, identify and clone the ZSLIT3 receptor.

Slit proteins have been characterized as chemorepellants in the neurological system, responsible for directing neurite growth and neuronal system organization. Slit protein polypeptides, agonists and antagonists can be used to modulate neurite growth and development and demarcate nervous system structures. Mutations deleting slit proteins result in axon projections in to inappropriate regions of the spinal cord. ZSLIT3 is expressed in various brain tissues and in spinal cord. ZSLIT3 polypeptides and ZSLIT3 antagonists, including anti-ZSLIT3 antibodies, would be useful as in treatment of peripheral neuropathies by increasing spinal cord and

sensory neurite outgrowth and patterning by acting as repulsive and attractive guidance cues to the developing sensory or motor neuron. Guidance cues serve to direct or constrain the pattern of neuron growth, channeling axons to their appropriate destination. In the absence of guidance cues neuron growth is random and unstructured. As such, ZSLIT3 polypeptides, agonists, and antagonists, including anti-ZSLIT3 antibodies, can be included in the therapeutic treatment for the regeneration and direction of neurite outgrowths following strokes, brain damage caused by head injuries and paralysis caused by spinal injuries. Application may also be made in treating neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, Parkinson's disease and peripheral neuropathies, or demyelinating diseases including multiple sclerosis, by directing neuronal outgrowths. Such an application would be repair of transected axons that are common in lesions of multiple sclerosis (Trapp et al., N. Engl. J. Med. 338:278-85, 1998). Similarly, application may also be made in treating neurodegenerative diseases or conditions as a result of exposure to neurotoxic chemical compounds.

ZSLIT3 is expressed in some non-neuronal tissues but likely influences the development and innervation of these tissues. G-Sema I and collapsin are hypothesized to act *in vivo* as repulsive or inhibitory molecules that prevent neighboring ventral motorneurons from innervating extra thoracic muscle. In other situations, G-Sema I and collapsin may also act as an attractive agent to promote innervation (Kolodkin,A.L. et al., Cell 75:1389-99, 1993). Similarly, ZSLIT3 polypeptides would be useful in directing neuronal development and innervation patterns in various tissues, and in organogenesis, for example in heart, lung, kidney, liver, and pancreas, and the like, by acting as a guidance cue and stimulating the formation of normal synaptic terminal arborizations, for example on a target muscle tissue.

Moreover, as slit proteins have been reported to play a role in the development of the central nervous system and other neuronal development, by acting as a restraining signal during such development. Similarly, ZSLIT3 would be useful in directing and defining the growth of developing neuronal and organ tissues, for example as, defining the margins or development of a particular organ or tissue. ZSLIT3 polypeptides would be useful in the defining and directing development of

various tissues and organs including those associated with heart, lung, kidney, liver, pancreas, muscle, fibroblasts, reproductive, endocrine and lymphatic tissues.

Moreover, In lung development, endodermally-derived lung buds undergo proliferation and branching in response to the mesenchyme. This is followed, later in development, by epithelium located at branch distal ends differentiating into pneumocytes. Soluble factors secreted by the mesenchyme affect epithelial branching. Secreted polypeptides expressed lung, such as ZSLIT3 molecules of the present invention, are useful in lung organogenesis and repair and as permissive or regulatory factors in this process. Moreover, ZSLIT3 molecules of the present invention can be used to diagnose and treat pulmonary diseases such as those associated with respiration and circulation, cystic fibrosis, asthma, emphysema, lung cancers and the like. Methods for using ZSLIT3 as a diagnostic and pharmaceutical composition are described herein.

Similarly, kidney development involves ductile growth and branching, and the formation of the renal epithelium. The latter differentiates onto the nephron. The collecting ducts are formed at this time as well. Secreted polypeptides expressed kidney, such as ZSLIT3 polypeptides of the present invention, are useful in kidney organogenesis and repair and in cases of kidney failure, for example, kidney failure associated with diabetes, or in pre-renal, renal, and post-renal complications (e.g., benign prostatic hyperplasia).

Moreover, as a secreted protein, ZSLIT3 may be a mediator of immunosuppression, in particular the activation and regulation of T lymphocytes. As such, ZSLIT3 polypeptides would be useful additions to therapies for treating immunodeficiencies. For example, ZSLIT3 can be useful in diagnosing and treating conditions where selective elimination of inappropriately activated T cells or other immune cells would be beneficial, such as in autoimmune diseases, in particular insulin dependent diabetes mellitus, rheumatoid arthritis and multiple sclerosis. Such polypeptides could be used to screen serum samples from patients suffering from such conditions in comparison to normal samples. Inappropriately activated T cells would include those specific for self-peptide/self-major histocompatibility complexes and those specific for non-self antigens from transplanted tissues. Use could also be made

of these polypeptides in blood screening for removal of inappropriately activated T cells before returning the blood to the donor. Those skilled in the art will recognize that conditions related to ZSLIT3 underexpression or overexpression may be amenable to treatment by therapeutic manipulation of ZSLIT3 protein levels.

ZSLIT3 polypeptides can be used *in vivo* as an anti-inflammatory, for inhibition of antigen in humoral and cellular immunity and for immunosuppression in graft and organ transplants. Methods of assessing ZSLIT3 pro- or anti-inflammatory effects are well known in the art.

ZSLIT3 polynucleotides and/or polypeptides can be used for regulating the proliferation and stimulation of a wide variety of cells, such as T cells, B cells, lymphocytes, peripheral blood mononuclear cells, fibroblasts and hematopoietic cells. ZSLIT3 polypeptides will also find use in mediating metabolic or physiological processes *in vivo*. Proliferation and differentiation can be measured *in vitro* using cultured cells. Suitable cell lines are available commercially from such sources as the American Type Culture Collection (Rockville, MD). Bioassays and ELISAs are available to measure cellular response to ZSLIT3, in particular are those which measure changes in cytokine production as a measure of cellular response (see for example, Current Protocols in Immunology ed. John Coligan et al., NIH, 1996). Also of interest are apoptosis assays, such as the DNA fragmentation assay described by Wiley et al. (Immunity, 3:673-82, 1995, and the cell death assay described by Pan et al., Science, 276:111-13, 1997). Assays to measure other cellular responses, including antibody isotype, monocyte activation, NK cell formation and antigen presenting cell function are also known. The ZSLIT3 polypeptides may also be used to stimulate lymphocyte development, such as during bone marrow transplantation and as therapy for some cancers.

In vivo response to ZSLIT3 polypeptides can also be measured by administering polypeptides of the claimed invention to the appropriate animal model. Well established animal models are available to test *in vivo* efficacy of ZSLIT3 polypeptides for certain disease states. In particular, ZSLIT3 polypeptides can be tested *in vivo* in a number of animal models of autoimmune disease, such as the NOD mice, a spontaneous model system for insulin-dependent diabetes mellitus (IDDM), to study

induction of non-responsiveness in the animal model. Administration of ZSLIT3 polypeptides prior to or after onset of disease can be monitored by assay of urine glucose levels in the NOD mouse. Alternatively, induced models of autoimmune disease, such as experimental allergic encephalitis (EAE), can be administered ZSLIT3 polypeptides. Administration in a preventive or intervention mode can be followed by monitoring the clinical symptoms of EAE. In addition, ZSLIT3 polypeptides can be tested *in vivo* in animal models for cancer, where suppression or apoptosis of introduced tumor cells can be monitored following administration of ZSLIT3.

As a ligand, the activity of ZSLIT3 polypeptide can be measured by a silicon-based biosensor microphysiometer which measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent physiologic cellular responses. An exemplary device is the Cytosensor™ Microphysiometer (Molecular Devices, Sunnyvale, CA). A variety of cellular responses, such as cell proliferation, ion transport, energy production, inflammatory response, regulatory and receptor activation, and the like, can be measured by this method. See, for example, McConnell et al., Science 257:1906-12, 1992; Pitchford et al., Meth. Enzymol. 228:84-108, 1997; Arimilli et al., J. Immunol. Meth. 212:49-59, 1998; Van Liefde et al., Eur. J. Pharmacol. 346:87-95, 1998. The microphysiometer can be used for assaying adherent or non-adherent eukaryotic or prokaryotic cells. By measuring extracellular acidification changes in cell media over time, the microphysiometer directly measures cellular responses to various stimuli, including ZSLIT3 polypeptide, its agonists, or antagonists. Preferably, the microphysiometer is used to measure responses of a ZSLIT3-responsive eukaryotic cell, compared to a control eukaryotic cell that does not respond to ZSLIT3 polypeptide. ZSLIT3-responsive eukaryotic cells comprise cells into which a receptor for ZSLIT3 has been transfected creating a cell that is responsive to ZSLIT3; or cells naturally responsive to ZSLIT3 such as cells derived from neurological, endrocrinological or tumor tissue. Differences, measured by a change, for example, an increase or diminution in extracellular acidification, in the response of cells exposed to ZSLIT3 polypeptide, relative to a control not exposed to ZSLIT3, are a direct measurement of ZSLIT3-modulated cellular responses. Moreover, such ZSLIT3-modulated responses can be

assayed under a variety of stimuli. Using the microphysiometer, there is provided a method of identifying agonists of ZSLIT3 polypeptide, comprising providing cells responsive to a ZSLIT3 polypeptide, culturing a first portion of the cells in the absence of a test compound, culturing a second portion of the cells in the presence of a test compound, and detecting a change, for example, an increase or diminution, in a cellular response of the second portion of the cells as compared to the first portion of the cells. The change in cellular response is shown as a measurable change extracellular acidification rate. Moreover, culturing a third portion of the cells in the presence of ZSLIT3 polypeptide and the absence of a test compound can be used as a positive control for the ZSLIT3-responsive cells, and as a control to compare the agonist activity of a test compound with that of the ZSLIT3 polypeptide. Moreover, using the microphysiometer, there is provided a method of identifying antagonists of ZSLIT3 polypeptide, comprising providing cells responsive to a ZSLIT3 polypeptide, culturing a first portion of the cells in the presence of ZSLIT3 and the absence of a test compound, culturing a second portion of the cells in the presence of ZSLIT3 and the presence of a test compound, and detecting a change, for example, an increase or a diminution in a cellular response of the second portion of the cells as compared to the first portion of the cells. The change in cellular response is shown as a measurable change extracellular acidification rate. Antagonists and agonists, for ZSLIT3 polypeptide, can be rapidly identified using this method.

Moreover, ZSLIT3 can be used to identify cells, tissues, or cell lines which respond to a ZSLIT3-stimulated pathway. The microphysiometer, described above, can be used to rapidly identify ligand-responsive cells, such as cells responsive to ZSLIT3 of the present invention. Cells can be cultured in the presence or absence of ZSLIT3 polypeptide. Those cells which elicit a measurable change in extracellular acidification in the presence of ZSLIT3 are responsive to ZSLIT3. Such cell lines, can be used to identify antagonists and agonists of ZSLIT3 polypeptide as described above.

ZSLIT3 polypeptides can also be used to identify inhibitors (antagonists) of its activity. ZSLIT3 antagonists include anti-ZSLIT3 antibodies and soluble ZSLIT3 receptors, as well as other peptidic and non-peptidic agents (including ribozymes). Test compounds are added to the assays disclosed herein to identify compounds that inhibit

the activity of ZSLIT3. In addition to those assays disclosed herein, samples can be tested for inhibition of ZSLIT3 activity within a variety of assays designed to measure receptor binding or the stimulation/inhibition of ZSLIT3-dependent cellular responses. For example, ZSLIT3-responsive cell lines can be transfected with a reporter gene construct that is responsive to a ZDMF-7-stimulated cellular pathway. Reporter gene constructs of this type are known in the art, and will generally comprise a ZSLIT3-DNA response element operably linked to a gene encoding an assayable protein, such as luciferase. DNA response elements can include, but are not limited to, cyclic AMP response elements (CRE), hormone response elements (HRE) insulin response element (IRE) (Nasrin et al., Proc. Natl. Acad. Sci. USA 87:5273-7, 1990) and serum response elements (SRE) (Shaw et al. Cell 56: 563-72, 1989). Cyclic AMP response elements are reviewed in Roestler et al., J. Biol. Chem. 263 (19):9063-6; 1988 and Habener, Molec. Endocrinol. 4 (8):1087-94; 1990. Hormone response elements are reviewed in Beato, Cell 56:335-44; 1989. Candidate compounds, solutions, mixtures or extracts are tested for the ability to inhibit the activity of ZSLIT3 on the target cells as evidenced by a decrease in ZSLIT3 stimulation of reporter gene expression. Assays of this type will detect compounds that directly block ZSLIT3 binding to cell-surface receptors, as well as compounds that block processes in the cellular pathway subsequent to receptor-ligand binding. In the alternative, compounds or other samples can be tested for direct blocking of ZSLIT3 binding to receptor using ZSLIT3 tagged with a detectable label (e.g., ^{125}I, biotin, horseradish peroxidase, FITC, and the like). Within assays of this type, the ability of a test sample to inhibit the binding of labeled ZSLIT3 to the receptor is indicative of inhibitory activity, which can be confirmed through secondary assays. Receptors used within binding assays may be cellular receptors or isolated, immobilized receptors.

ZSLIT3 antagonists would find use to modulate or down regulate one or more detrimental biological processes in cells, tissues and/or biological fluids, such as over-responsiveness, unregulated or inappropriate growth, and inflammation or allergic reaction. ZSLIT3 antagonists would have beneficial therapeutic effect in diseases where the inhibition of activation of certain B lymphocytes and/or T cells would be effective. In particular, such diseases would include autoimmune diseases, such as

multiple sclerosis, insulin-dependent diabetes and systemic lupus erythematosus. Also, benefit would be derived from using ZSLIT3 antagonists for chronic inflammatory and infective diseases. Antagonists could be used to dampen or inactivate ZSLIT3 during activated immune response.

The activity of slit protein polypeptides, agonists, antagonists and antibodies of the present invention can be measured, and compounds screened to identify agonists and antagonists, using a variety of assays, such as assays that measure axon guidance and growth and neuronal migration. Such assays are suitable to assess the axon guidance and growth and neuronal migration activity of the ZSLIT3 polypeptides of the present invention. Of particular interest are assays that indicate changes in neuron growth patterns, see for example, Hastings, WIPO Patent Application No:97/29189 and Walter et al., Development 101:685-96, 1987. Assays to measure the effects of proteins on neuron growth are well known in the art. For example, the C assay (see for example, Raper and Kapfhammer, Neuron 4:21-9, 1990 and Luo et al., Cell 75:217-27, 1993), and other chemorepulsion and collapsing assays (see, e.g., Ba-Charvet, K.T.N, et al., Neuron 22:463-473, 1999) can be used to determine collapsing activity slit proteins on growing neurons. In addition slit protein chemorepulsion of neuronal migration can be measured (see, Hu, H., Neuron 23:703-711, 1999; Wu, W. Nature 400:331-336, 1999). Other methods which assess protein induced inhibition of neurite extension or divert such extension are also known, see Goodman, Annu. Rev. Neurosci. 19:341-77, 1996. Conditioned media from cells expressing a slit protein, such as ZSLIT3, a slit protein agonist or slit protein antagonist, or aggregates of such cells, can by placed in a gel matrix near suitable neural cells, such as dorsal root ganglia (DRG) or sympathetic ganglia explants, which have been co-cultured with nerve growth factor. Compared to control cells, ZSLIT3 protein-induced changes in neuron growth can be measured (see, for example, Messersmith et al., Neuron 14:949-59, 1995; Puschel et al., Neuron 14:941-8, 1995); or in a motor neuron repulsion assay (Brose, K. et al., Cell 96:795-806, 1999). Likewise neurite outgrowth can be measured using neuronal cell suspensions grown in the presence of molecules of the present invention. See for example, O'Shea et al., Neuron 7:231-7, 1991 and DeFreitas et al., Neuron 15:333-43, 1995.

Also available are assay systems that use a ligand-binding receptor (or an antibody, one member of a complement/anti-complement pair) or a binding fragment thereof, and a commercially available biosensor instrument (BIAcore™, Pharmacia Biosensor, Piscataway, NJ). As used herein, "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^9$ M^{-1}. Such receptor, antibody, member of a complement/anti-complement pair or fragment is immobilized onto the surface of a receptor chip. Use of this instrument is disclosed by Karlsson, J. Immunol. Methods 145:229-40, 1991 and Cunningham and Wells, J. Mol. Biol. 234:554-63, 1993. A receptor, antibody, member or fragment is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within the flow cell. A test sample is passed through the cell. If a ligand, epitope, or opposite member of the complement/anti-complement pair is present in the sample, it will bind to the immobilized receptor, antibody or member, respectively, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding. Ligand-binding receptor polypeptides can also be used within other assay systems known in the art. Such systems include Scatchard analysis for determination of binding affinity (see, Scatchard, Ann. NY Acad. Sci. 51: 660-72, 1949) and calorimetric assays (Cunningham et al., Science 253:545-8, 1991; Cunningham et al., Science 245:821-5, 1991).

An *in vivo* approach for assaying proteins of the present invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, retroviruses, vaccinia virus, and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer

vector for delivery of heterologous nucleic acid (for review, see T.C. Becker et al., Meth. Cell Biol. 43:161-89, 1994; and J.T. Douglas and D.T. Curiel, Science & Medicine 4:44-53, 1997). The adenovirus system offers several advantages: (i) adenovirus can accommodate relatively large DNA inserts; (ii) can be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) can be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. Also, because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection.

Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene has been deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell (the human 293 cell line is exemplary). When intravenously administered to intact animals, adenovirus primarily targets the liver. If the adenoviral delivery system has an E1 gene deletion, the virus cannot replicate in the host cells. However, the host's tissue (e.g., liver) will express and process (and, if a secretory signal sequence is present, secrete) the heterologous protein. Secreted proteins will enter the circulation in the highly vascularized liver, and effects on the infected animal can be determined.

Moreover, adenoviral vectors containing various deletions of viral genes can be used in an attempt to reduce or eliminate immune responses to the vector. Such adenoviruses are E1 deleted, and in addition contain deletions of E2A or E4 (Lusky, M. et al., J. Virol. 72:2022-2032, 1998; Raper, S.E. et al., Human Gene Therapy 9:671-679, 1998). In addition, deletion of E2b is reported to reduce immune responses (Amalfitano, A. et al., J. Virol. 72:926-933, 1998). Moreover, by deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses where all viral genes are deleted are particularly advantageous for insertion of large inserts of heterologous DNA. For review, see Yeh, P. and Perricaudet, M., FASEB J. 11:615-623, 1997.

The adenovirus system can also be used for protein production *in vitro*. By culturing adenovirus-infected non-293 cells under conditions where the cells are not

rapidly dividing, the cells can produce proteins for extended periods of time. For instance, BHK cells are grown to confluence in cell factories, then exposed to the adenoviral vector encoding the secreted protein of interest. The cells are then grown under serum-free conditions, which allows infected cells to survive for several weeks without significant cell division. Alternatively, adenovirus vector infected 293 cells can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (See Garnier et al., Cytotechnol. 15:145-55, 1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant, lysate, or membrane fractions depending on the disposition of the expressed protein in the cell. Within the infected 293 cell production protocol, non-secreted proteins may also be effectively obtained.

. is a progressive and dynamic process, beginning with pluripotent stem cells and ending with terminally differentiated cells. Pluripotent stem cells that can regenerate without commitment to a lineage express a set of differentiation markers that are lost when commitment to a cell lineage is made. Progenitor cells express a set of differentiation markers that may or may not continue to be expressed as the cells progress down the cell lineage pathway toward maturation. Differentiation markers that are expressed exclusively by mature cells are usually functional properties such as cell products, enzymes to produce cell products, and receptors. The stage of a cell population's differentiation is monitored by identification of markers present in the cell population. Myocytes, osteoblasts, adipocytes, chrondrocytes, fibroblasts and reticular cells are believed to originate from a common mesenchymal stem cell (Owen et al., Ciba Fdn. Symp. 136:42-46, 1988). Markers for mesenchymal stem cells have not been well identified (Owen et al., J. of Cell Sci. 87:731-738, 1987), so identification is usually made at the progenitor and mature cell stages. The novel polypeptides of the present invention may be useful for studies to isolate mesenchymal stem cells and myocyte or other progenitor cells, both *in vivo* and *ex vivo*.

There is evidence to suggest that factors that stimulate specific cell types down a pathway towards terminal differentiation or dedifferentiation affect the entire cell population originating from a common precursor or stem cell. Thus, the present invention includes stimulating or inhibiting the proliferation of myocytes, smooth

muscle cells, osteoblasts, adipocytes, chrondrocytes, neuronal and endothelial cells. Molecules of the present invention for example, may while stimulating proliferation or differentiation of cardiac myocytes, inhibit proliferation or differentiation of adipocytes, by virtue of the affect on their common precursor/stem cells. Thus molecules of the present invention may have use in inhibiting chondrosarcomas, atherosclerosis, restenosis and obesity.

Assays measuring differentiation include, for example, measuring cell markers associated with stage-specific expression of a tissue, enzymatic activity, functional activity or morphological changes (Watt, FASEB, 5:281-284, 1991; Francis, Differentiation 57:63-75, 1994; Raes, Adv. Anim. Cell Biol. Technol. Bioprocesses, 161-171, 1989; all incorporated herein by reference). Alternatively, ZSLIT3 polypeptide itself can serve as an additional cell-surface or secreted marker associated with stage-specific expression of a tissue. As such, direct measurement of ZSLIT3 polypeptide, or its loss of expression in a tissue as it differentiates, can serve as a marker for differentiation of tissues.

Similarly, direct measurement of ZSLIT3 polypeptide, or its loss of expression in a tissue can be determined in a tissue or cells as they undergo tumor progression. Increases in invasiveness and motility of cells, or the gain or loss of expression of ZSLIT3 in a pre-cancerous or cancerous condition, in comparison to normal tissue, can serve as a diagnostic for transformation, invasion and metastasis in tumor progression. As such, knowledge of a tumor's stage of progression or metastasis will aid the physician in choosing the most proper therapy, or aggressiveness of treatment, for a given individual cancer patient. Methods of measuring gain and loss of expression (of either mRNA or protein) are well known in the art and described herein and can be applied to ZSLIT3 expression. For example, appearance or disappearance of polypeptides that regulate cell motility can be used to aid diagnosis and prognosis of prostate cancer (Banyard, J. and Zetter, B.R., Cancer and Metast. Rev. 17:449-458, 1999). As an effector of cell motility and growth, ZSLIT3 gain or loss of expression may serve as a diagnostic for prostate and other cancers. In addition, ZSLIT3 is expressed in cancerous glioblastoma tissue, and osteogenic sarcoma, breast, and intestinal cancer cells. As such, polynucleotides, polypeptides, and anti-ZSLIT3

antibodies and binding partners can be used to detect these cancers in biopsies, tissue samples, histologic sections, and *in vivo*. Moreover, analogous to the prostate specific antigen (PSA), as a naturally-expressed testicular marker, increased levels of ZSLIT3 polypeptides, or anti-ZSLIT3 antibodies in a patient, relative to a normal control can be indicative of brain, testis and ovarian diseases, such as brain, testis and ovarian cancer, and possibly lung, live, kidney, prostate and pancreatic cancers (See, e.g., Mulders, TMT, et al., Eur. J. Surgical Oncol. 16:37-41, 1990). Moreover, as ZSLIT3 expression appears to be restricted to specific human tissues, lack of ZSLIT3 expression in those tissues or strong ZSLIT3 expression in tissues where ZSLIT3 is not normally expressed, would serve as a diagnostic of an abnormality in the cell or tissue type, of invasion or metastasis of cancerous testicular tissues into non-testicular tissue, and could aid a physician in directing further testing or investigation, or aid in directing therapy.

In addition, as ZSLIT3 has high levels of testis and ovary-specific expression, polynucleotide probes, anti-ZSLIT3 antibodies, and detection the presence of ZSLIT3 polypeptides in tissue can be used to assess whether these tissues are present, for example, after surgery involving the excision of a diseased or cancerous testis or ovarian tissue. As such, the polynucleotides, polypeptides, and antibodies of the present invention can be used as an aid to determine whether all such tissue is excised after surgery, for example, after surgery for cancer. In such instances, it is especially important to remove all potentially diseased tissue to maximize recovery from the cancer, and to minimize recurrence. Preferred embodiments include fluorescent, radiolabeled, or calorimetrically labeled anti-ZSLIT3 antibodies and ZSLIT3 polypeptide binding partners, that can be used histologically or *in situ*.

Moreover, the activity and effect of ZSLIT3 on tumor progression and metastasis can be measured *in vivo*. Several syngeneic mouse models have been developed to study the influence of polypeptides, compounds or other treatments on tumor progression. In these models, tumor cells passaged in culture are implanted into mice of the same strain as the tumor donor. The cells will develop into tumors having similar characteristics in the recipient mice, and metastasis will also occur in some of the models. Appropriate tumor models for our studies include the Lewis lung carcinoma (ATCC No. CRL-1642) and B16 melanoma (ATCC No. CRL-6323),

amongst others. These are both commonly used tumor lines, syngeneic to the C57BL6 mouse, that are readily cultured and manipulated *in vitro*. Tumors resulting from implantation of either of these cell lines are capable of metastasis to the lung in C57BL6 mice. The Lewis lung carcinoma model has recently been used in mice to identify an inhibitor of angiogenesis (O'Reilly MS, et al. Cell 79: 315-328,1994). C57BL6/J mice are treated with an experimental agent either through daily injection of recombinant protein, agonist or antagonist or a one time injection of recombinant adenovirus. Three days following this treatment, 10^5 to 10^6 cells are implanted under the dorsal skin. Alternatively, the cells themselves may be infected with recombinant adenovirus, such as one expressing ZSLIT3, before implantation so that the protein is synthesized at the tumor site or intracellularly, rather than systemically. The mice normally develop visible tumors within 5 days. The tumors are allowed to grow for a period of up to 3 weeks, during which time they may reach a size of 1500 - 1800 mm^3 in the control treated group. Tumor size and body weight are carefully monitored throughout the experiment. At the time of sacrifice, the tumor is removed and weighed along with the lungs and the liver. The lung weight has been shown to correlate well with metastatic tumor burden. As an additional measure, lung surface metastases are counted. The resected tumor, lungs and liver are prepared for histopathological examination, immunohistochemistry, and *in situ* hybridization, using methods known in the art and described herein. The influence of the expressed polypeptide in question, e.g., ZSLIT3, on the ability of the tumor to recruit vasculature and undergo metastasis can thus be assessed. In addition, aside from using adenovirus, the implanted cells can be transiently transfected with ZSLIT3. Use of stable ZSLIT3 transfectants as well as use of induceable promoters to activate ZSLIT3 expression *in vivo* are known in the art and can be used in this system to assess ZSLIT3 induction of metastasis. Moreover, purified ZSLIT3 or ZSLIT3 conditioned media can be directly injected in to this mouse model, and hence be used in this system. For general reference see, O'Reilly MS, et al. Cell 79:315-328, 1994; and Rusciano D, et al. Murine Models of Liver Metastasis. Invasion Metastasis 14:349-361, 1995.

Since ZSLIT3 is expressed in heart, it could be useful as modulator blood pressure, muscle tension or and osmotic balance. For example, blood pressure

modification is important in situations such as heart attack, stroke, traumatic shock, surgery, and any number of bleeding complications. As a modulator of blood pressure, muscle tension or and osmotic balance, ZSLIT3 may modulate contractility in the organ systems and tissues that it effects. Thus, The activity of molecules of the present invention can be measured using a variety of assays that measure cell contractility and discussed below. Such assays are well known in the art.

ZSLIT3 is expressed in tissues that contract, thus it can be used to modulate such contractile tissues. For example contractile tissues where ZSLIT3 is expressed include, tissues in testis, e.g., vas deferens; prostate tissues; gastrointestinal tissues, e.g., colon and small intestine; and heart, lung, and ovary, and may include uterus. The effects of ZSLIT3 polypeptide, its antagonists and agonists, on tissue contractility can be measured *in vitro* using a tensiometer with or without electrical field stimulation. Such assays are known in the art and can be applied to tissue samples, such as aortic rings, vas deferens, ilium, uterine and other contractile tissue samples, as well as to organ systems, such as atria, and can be used to determine whether ZSLIT3 polypeptide, its agonists or antagonists, enhance or depress contractility. Molecules of the present invention are hence useful for treating dysfunction associated with contractile tissues or can be used to suppress or enhance contractility *in vivo*. As such, molecules of the present invention have utility in treating cardiovascular disease, infertility, *in vitro* fertilization, birth control, treating impotence or other male reproductive dysfunction, as well as inducing birth.

The effect of the ZSLIT3 polypeptides, antagonists and agonists of the present invention on contractility of tissues including uterus, prostate, testis, gastrointestinal tissues, and heart can be measured in a tensiometer that measures contractility and relaxation in tissues. See, Dainty et al., J. Pharmacol. 100:767, 1990; Rhee et al., Neurotox. 16: 179, 1995; Anderson, M.B., Endocrinol. 114:364-368, 1984; and Downing, S.J. and Sherwood, O.D, Endocrinol. 116:1206-1214, 1985. For example, measuring vasodilatation of aortic rings is well known in the art. Briefly, aortic rings are taken from 4 month old Sprague Dawley rats and placed in a buffer solution, such as modified Krebs solution (118.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2.2H_2O$, 24.8 mM $NaHCO_3$ and 10 mM

glucose). One of skill in the art would recognize that this method can be used with other animals, such as rabbits, other rat strains, Guinea pigs, and the like. The rings are then attached to an isometric force transducer (Radnoti Inc., Monrovia, CA) and the data recorded with a Ponemah physiology platform (Gould Instrument systems, Inc., Valley View, OH) and placed in an oxygenated (95% O_2, 5% CO_2) tissue bath containing the buffer solution. The tissues are adjusted to 1 gram resting tension and allowed to stabilize for about one hour before testing. The integrity of the rings can be tested with norepinepherin (Sigma Co., St. Louis, MO) and Carbachol, a muscarinic acetylcholine agonist (Sigma Co.). After integrity is checked, the rings are washed three times with fresh buffer and allowed to rest for about one hour. To test a sample for vasodilatation, or relaxation of the aortic ring tissue, the rings are contracted to two grams tension and allowed to stabilize for fifteen minutes. A ZSLIT3 polypeptide sample is then added to 1, 2 or 3 of the 4 baths, without flushing, and tension on the rings recorded and compared to the control rings containing buffer only. Enhancement or relaxation of contractility by ZSLIT3 polypeptides, their agonists and antagonists is directly measured by this method, and it can be applied to other contractile tissues such as gastrointestinal , lung, prostate, and testis.

The activity of molecules of the present invention can be measured using a variety of assays that measure stimulation of gastrointestinal cell contractility, modulation of nutrient uptake and/or secretion of digestive enzymes. Of particular interest are changes in contractility of smooth muscle cells. For example, the contractile response of segments of mammalian duodenum or other gastrointestinal smooth muscles tissue (Depoortere et al., J. Gastrointestinal Motility 1:150-159, 1989, incorporated herein by reference). An exemplary *in vivo* assay uses an ultrasonic micrometer to measure the dimensional changes radially between commissures and longitudinally to the plane of the valve base (Hansen et al., Society of Thoracic Surgeons 60:S384-390, 1995).

Gastric motility is generally measured in the clinical setting as the time required for gastric emptying and subsequent transit time through the gastrointestinal tract. Gastric emptying scans are well known to those skilled in the art, and briefly, comprise use of an oral contrast agent, such as barium, or a radiolabeled meal. Solids

and liquids can be measured independently. A test food or liquid is radiolabeled with an isotope (e.g. ^{99m}Tc), and after ingestion or administration, transit time through the gastrointestinal tract and gastric emptying are measured by visualization using gamma cameras (Meyer et al., Am. J. Dig. Dis. 21:296, 1976; Collins et al., Gut 24:1117, 1983; Maughan et al., Diabet. Med. 13 9 Supp. 5:S6-10, 1996 and Horowitz et al., Arch. Intern. Med. 145:1467-1472, 1985). These studies may be performed before and after the administration of a pro-motility agent to quantify the efficacy of the drug.

The polypeptides, antagonists, agonists, nucleic acid and/or antibodies of the present invention can also be used in treatment of disorders associated with gastrointestinal cell contractility, secretion of digestive enzymes and acids, gastrointestinal motility, recruitment of digestive enzymes; inflammation, particularly as it affects the gastrointestinal system; reflux disease and regulation of nutrient absorption. Specific conditions that will benefit from treatment with molecules of the present invention include, but are not limited to, diabetic gastroparesis, post-surgical gastroparesis, vagotomy, chronic idiopathic intestinal pseudo-obstruction and gastroesophageal reflux disease. Additional uses include, gastric emptying for radiological studies, stimulating gallbladder contraction and antrectomy.

The motor and neurological affects of molecules of the present invention make it useful for treatment of obesity and other metabolic disorders where neurological feedback modulates nutritional absorption. The molecules of the present invention are useful for regulating satiety, glucose absorption and metabolism, and neuropathy-associated gastrointestinal disorders. Molecules of the present invention are also useful as additives to anti-hypoglycemic preparations containing glucose and as adsorption enhancers for oral drugs which require fast nutrient action. Additionally, molecules of the present invention can be used to stimulate glucose-induced insulin release.

Moreover, tissues in which the polypeptides of the present invention are expressed are comprised in part of epithelial cells where ZSLIT3 polypeptides, agonists or antagonists thereof may be therapeutically useful for promoting wound healing. To verify the presence of this capability in ZSLIT3 polypeptides, agonists or antagonists of the present invention, such ZSLIT3 polypeptides, agonists or antagonists are evaluated

with respect to their ability to facilitate wound healing according to procedures known in the art. If desired, ZSLIT3 polypeptide performance in this regard can be compared to growth factors, such as EGF, NGF, TGF-α, TGF-β, insulin, IGF-I, IGF-II, fibroblast growth factor (FGF) and the like. Moreover, the effects of ZSLIT3 polypeptides, agonists or antagonists thereof can be evaluated with respect to their ability to enhance wound contractility involved in wound healing. In addition, ZSLIT3 polypeptides or agonists or antagonists thereof may be evaluated in combination with one or more growth factors to identify synergistic effects.

The molecules of the present invention are useful as components of defined cell culture media, as described herein, and may be used alone or in combination with other cytokines and hormones to replace serum that is commonly used in cell culture. Molecules of the present invention are particularly useful in specifically promoting the growth, development, differentiation, and/or maturation of ovarian cells in culture, and may also prove useful in the study of the ovarian cycle, reproductive function, ovarian and testicular cell-cell interactions, sperm capacitation and fertilization.

In addition, the present invention also provides methods for studying steroidogenesis and steroid hormone secretion. Such methods generally comprise incubating ovarian cells in culture medium comprising ZSLIT3 polypeptides, monoclonal antibodies, agonists or antagonists thereof with and without gonadotropins and/or steroid hormones, and subsequently observing protein and steroid secretion. Exemplary gonadotropin hormones include luteinizing hormone and follicle stimulating hormone (Rouillier et al., Mol. Reprod. Dev. 50:170-7, 1998). Exemplary steroid hormones include estradiol, androstenedione, and progesterone. Effects of ZSLIT3 on steroidogenesis or steroid secretion can be determined by methods known in the art, such as radioimmunoassay (to detect levels of estradiol, androstenedione, progesterone, and the like), and immunoradiometric assay (IRMA).

Molecules expressed in the ovary, testis and prostate, such as ZSLIT3 polypeptide which is specifically highly expressed in testis and ovary, and which may modulate hormones, hormone receptors, growth factors, or cell-cell interactions, of the reproductive cascade or are involved in oocyte or ovarian development,

spermatogenesis, or the like, would be useful as markers for cancer of reproductive organs and as therapeutic agents for hormone-dependent cancers, by inhibiting hormone-dependent growth and/or development of tumor cells. Human reproductive system cancers such as ovarian, uterine, cervical, testicular and prostate cancers are common. Moreover, receptors for steroid hormones involved in the reproductive cascade are found in human tumors and tumor cell lines (breast, prostate, endometrial, ovarian, kidney, and pancreatic tumors) (Kakar et al., Mol. Cell. Endocrinol., 106:145-49, 1994; Kakar and Jennes, Cancer Letts., 98:57-62, 1995). Thus, expression of ZSLIT3 in reproductive tissues suggests that polypeptides of the present invention would be useful in diagnostic methods for the detection and monitoring of reproductive cancers.

Diagnostic methods of the present invention involve the detection of ZSLIT3 polypeptides in the serum or tissue biopsy of a patient undergoing analysis of reproductive function or evaluation for possible reproductive cancers, e.g., uterine, testicular or prostate cancer. Such polypeptides can be detected using immunoassay techniques and antibodies, described herein, that are capable of recognizing ZSLIT3 polypeptide epitopes. More specifically, the present invention contemplates methods for detecting ZSLIT3 polypeptides comprising:

exposing a test sample potentially containing ZSLIT3 polypeptides to an antibody attached to a solid support, wherein said antibody binds to a first epitope of a ZSLIT3 polypeptide;

washing the immobilized antibody-polypeptide to remove unbound contaminants;

exposing the immobilized antibody-polypeptide to a second antibody directed to a second epitope of a ZSLIT3 polypeptide, wherein the second antibody is associated with a detectable label; and

detecting the detectable label. Altered levels of ZSLIT3 polypeptides in a test sample, such as serum sweat, saliva, biopsy, and the like, can be monitored as an indication of reproductive function or of reproductive cancer or disease, when compared against a normal control. Such methods detecting altered levels of ZSLIT3 polypeptides in a test sample can also be used to detect cancers in other organs or

tissues as well. Similarly, detecting altered levels of ZSLIT3 mRNA expression in a test sample can be used as a diagnostic. For example, ZSLIT3 is expressed in several cancer cell lines (See, Example 3). For example, a PCR-based method, such as that shown in Example 3 can be used as a cancer or disease diagnostic, as described above.

Additional methods using probes or primers derived, for example, from the nucleotide sequences disclosed herein can also be used to detect ZSLIT3 expression in a patient sample, such as a blood, saliva, sweat, biopsy, tissue sample, or the like. For example, probes can be hybridized to tumor tissues and the hybridized complex detected by *in situ* hybridization. ZSLIT3 sequences can also be detected by PCR amplification using cDNA generated by reverse translation of sample mRNA as a template (PCR Primer A Laboratory Manual, Dieffenbach and Dveksler, eds., Cold Spring Harbor Press, 1995). When compared with a normal control, both increases or decreases of ZSLIT3 expression in a patient sample, relative to that of a control, can be monitored and used as an indicator or diagnostic for disease.

The polypeptides, antagonists, agonists, nucleic acid and/or antibodies of the present invention may be used in treatment of disorders associated with gonadal development, pregnancy, pubertal changes, menopause, ovarian cancer, fertility, ovarian function, polycystic ovarian syndrome, uterine cancer, endometriosis, libido, mylagia and neuralgia associated with reproductive phenomena, male sexual dysfunction, impotency, prostate cancer, testicular cancer, colon and stomach cancer, gastrointestinal mobility and dysfunction. The molecules of the present invention may used to modulate or to treat or prevent development of pathological conditions in such diverse tissue as neuronal tissues, heart and ovary. In particular, certain syndromes or diseases may be amenable to such diagnosis, treatment or prevention. Moreover, natural functions, such as embryo implantation or spermatogenesis, may be suppressed or controlled for use in birth control by molecules of the present invention.

ZSLIT3 polypeptide is expressed in the ovary and may have additional biological activity independent of prostate or testis function, as described herein. Oogenesis is the process by which a diploid stem cell proceeds through multiple stages of differentiation, culminating in the formation of a terminally differentiated cell with a unique function, an oocyte. Unlike spermatogenesis, which begins at puberty and

continues on through the life of a male, oogenesis begins during fetal development and by birth, a female's entire supply of primary oocytes are stored in the ovaries in primordial follicles and await maturation and release.

In the adult ovary, folliculogenesis starts when the follicles enter the growth phase. Early growing follicles undergo a dramatic process of cellular proliferation and differentiation. The classic control of ovarian function by luteinizing hormone (LH) and follicle stimulating hormone (FSH) is now thought to include the action of a variety of molecules that act to promote cell-cell interactions between cells of the follicle. For review, see Gougeon, A., Endocrine Rev. 17:121-155, 1996. Hence, the mechanisms for controlling ovarian folliculogenesis and dominant follicle selection are still under investigation. As ZSLIT3 is expressed in the ovary, it may serve a role in modulating ovarian function by regulating folliculogenesis and dominant follicle selection, by affecting proliferation or differentiation of follicular cells, affecting cell-cell interactions, modulating hormones involved in the process, and the like.

The ovarian cycle in mammals includes the growth and maturation of follicles, followed by ovulation and transformation of follicles into corpea lutea. The physiological events in the ovarian cycle are dependent on interactions between hormones and cells within the hypothalamic-pituitary-ovarian axis, including gonadotropin releasing hormone (GnRH), LH, and FSH. In addition, estradiol, synthesized in the follicle, primes the hypothalamic-pituitary axis and is required for the mid-cycle surge of gonadotropin that stimulates the resumption of oocyte meiosis and leads to ovulation and subsequent extrusion of an oocyte from the follicle. This gonadotropin surge also promotes the differentiation of the follicular cells from secreting estradiol to secreting progesterone. Progesterone, secreted by the corpus luteum, is needed for uterine development required for the implantation of fertilized oocytes. The central role of hypothalamic-pituitary-gonadal hormones in the ovarian cycle and reproductive cascade, and the role of sex steroids on target tissues and organs, e.g., uterus, breast, adipose, bones and liver, has made modulators of their activity desirable for therapeutic applications. Such applications include treatments for precocious puberty, endometriosis, uterine leiomyomata, hirsutism, infertility, pre menstrual syndrome (PMS), amenorrhea, and as contraceptive agents.

ZSLIT3 polypeptides, agonists and antagonists which modulate the actions of such hormones can be of therapeutic value. Such molecules can also be useful for modulating steroidogenesis, both *in vivo* and *in vitro*, and modulating aspects of the ovarian cycle such as oocyte maturation, ovarian cell-cell interactions, follicular development and rupture, luteal function, menstruation, and promoting uterine implantation of fertilized oocytes. Molecules which modulate hormone action can be beneficial therapeutics for use prior to or at onset of puberty, or in adult women. For example, puberty in females is marked by an establishment of feed-back loops to control hormone levels and hormone production. Abnormalities resulting from hormone imbalances during puberty have been observed and include precocious puberty, where pubertal changes occur in females prior to the age of 8. Hormone-modulating molecules, can be used, in this case, to suppress hormone secretion and delay onset of puberty.

The level and ratio of gonadotropin and steroid hormones can be used to assess the existence of hormonal imbalances associated with diseases, as well as determine whether normal hormonal balance has been restored after administration of a therapeutic agent. Determination of estradiol, progesterone, LH, and FSH, for example, from serum is known by one of skill in the art. Such assays can be used to monitor the hormone levels after administration of ZSLIT3 *in vivo*, or in a transgenic mouse model where the ZSLIT3 gene is expressed or the murine ortholog is deleted. Thus, as a hormone-modulating molecule, ZSLIT3 polypeptides can have therapeutic application for treating, for example, breakthrough menopausal bleeding, as part of a therapeutic regime for pregnancy support, or for treating symptoms associated with polycystic ovarian syndrome (PCOS), endometriosis, PMS and menopause. In addition, other *in vivo* rodent models are known in the art to assay effects of ZSLIT3 polypeptide on, for example, polycystic ovarian syndrome (PCOS).

Proteins of the present invention may also be used in applications for enhancing fertilization during assisted reproduction in humans and in animals. Such assisted reproduction methods are known in the art and include artificial insemination, *in vitro* fertilization, embryo transfer, and gamete intrafallopian transfer. Such methods are useful for assisting those who may have physiological or metabolic disorders that

prevent or impede natural conception. Such methods are also used in animal breeding programs, e.g., for livestock, racehorses, domestic and wild animals, and could be used as methods for the creation of transgenic animals. ZSLIT3 polypeptides could be used in the induction of ovulation, either independently or in conjunction with a regimen of gonadotropins or agents such as clomiphene citrate or bromocriptine (Speroff et al., Induction of ovulation, Clinical Gynecologic Endocrinology and Infertility, 5th ed., Baltimore, Williams & Wilkins, 1994). As such, proteins of the present invention can be administered to the recipient prior to fertilization or combined with the sperm, an egg or an egg-sperm mixture prior to *in vitro* or *in vivo* fertilization. Such proteins can also be mixed with oocytes prior to cryopreservation to enhance viability of the preserved oocytes for use in assisted reproduction.

The ZSLIT3 polypeptides, agonists and antagonists of the present invention may be directly used as or incorporated into therapies for treating reproductive disorders. Disorders such as luteal phase deficiency would benefit from such therapy (Soules, "Luteal phase deficiency: A subtle abnormality of ovulation" in, Infertility: Evaluation and Treatment, Keye et al., eds., Philadelphia, WB Saunders, 1995). Moreover, administration of gonadotropin-releasing hormone is shown to stimulate reproductive behavior (Riskin and Moss, Res. Bull. 11:481-5, 1983; Kadar et al., Physiol. Behav. 51:601-5, 1992 and Silver et al., J. Neruoendocrin. 4:207-10, 199; King and Millar, Cell. Mol. Neurobiol., 15:5-23, 1995). Given the high prevalence of sexual dysfunction and impotence in humans, molecules, such as ZSLIT3, which may modulate or enhance gonadotropin activity can find application in developing treatments for these conditions. Conversely, polypeptides of the present invention, their antagonists or agonists can be used to inhibit normal reproduction in the form of birth control, for example, by decreasing spermatogenesis or preventing uterine implantation of a fertilized egg.

The ZSLIT3 polypeptides of the present invention can be used to study ovarian cell proliferation, maturation, and differentiation, i.e., by acting as a luteinizing agent that converts granulosa cells from estradiol to progesterone-producing cells. Such methods of the present invention generally comprise incubating granulosa cells, theca cells, oocytes or a combination thereof, in the presence and absence of ZSLIT3

polypeptide, monoclonal antibody, agonist or antagonist thereof and observing changes in cell proliferation, maturation and differentiation. See for example, Basini et al.,(J. Rep. Immunol. 37:139-53, 1998); Duleba et al.,(Fert. Ster. 69:335-40, 1998); and Campbell, B.K. et al., J. Reprod. and Fert. 112:69-77, 1998).

Molecules expressed in the ovary, testis and prostate, such as ZSLIT3 polypeptide which is specifically highly expressed in testis and ovary, and which may modulate hormones, hormone receptors, growth factors, or cell-cell interactions, of the reproductive cascade or are involved in oocyte or ovarian development, spermatogenesis, or the like, would be useful as markers for cancer of reproductive organs and as therapeutic agents for hormone-dependent cancers, by inhibiting hormone-dependent growth and/or development of tumor cells. Human reproductive system cancers such as ovarian, uterine, cervical, testicular and prostate cancers are common. Moreover, receptors for steroid hormones involved in the reproductive cascade are found in human tumors and tumor cell lines (breast, prostate, endometrial, ovarian, kidney, and pancreatic tumors) (Kakar et al., Mol. Cell. Endocrinol., 106:145-49, 1994; Kakar and Jennes, Cancer Letts., 98:57-62, 1995). Thus, expression of ZSLIT3 in reproductive tissues suggests that polypeptides of the present invention would be useful in diagnostic methods for the detection and monitoring of reproductive cancers.

Diagnostic methods of the present invention involve the detection of ZSLIT3 polypeptides in the serum or tissue biopsy of a patient undergoing analysis of reproductive function or evaluation for possible reproductive tissue cancers, e.g., ovarian, uterine, testicular, osteogenic sarcoma, intestinal carcinoma, breast carcinoma, glioblastoma or prostate cancer. Such polypeptides can be detected using immunoassay techniques and antibodies, described herein that are capable of recognizing ZSLIT3 polypeptide epitopes. When compared with a normal control, both increases or decreases of ZSLIT3 expression in a patient sample, relative to that of a control, can be monitored and used as an indicator or diagnostic for disease. The present invention contemplates methods for detecting ZSLIT3 polypeptides and mRNA, described above, and can be applied to the detection and monitoring of reproductive disease, for example, in the testis and ovary.

The ZSLIT3 polypeptides, antagonists of agonists, of the present invention can also modulate sperm capacitation. Before reaching the oocyte or egg and initiating an egg-sperm interaction, the sperm must be activated. The sperm undergo a gradual capacitation, lasting up to 3 or 4 hours *in vitro*, during which the plasma membrane of the sperm head and the outer acrosomal membrane fuse to form vesicles that facilitate the release of acrosomal enzymes. The acrosomal membrane surrounds the acrosome or acrosomal cap which is located at the anterior end of the nucleus in the sperm head. In order for the sperm to fertilize egg the sperm must penetrate the oocyte. To enable this process the sperm must undergo acrosomal exocytosis, also known as the acrosomal reaction, and release the acrosomal enzymes in the vicinity of the oocyte. These enzymes enable the sperm to penetrate the various oocyte layers, (the cumulus oophorus, the corona radiata and the zona pellucida). The released acrosomal enzymes include hyaluronidase and proacrosin, in addition to other enzymes such as proteases. During the acrosomal reaction, proacrosin is converted to acrosin, the active form of the enzyme, which is required for and must occur before binding and penetration of the zona pellucida is possible. A combination of the acrosomal lytic enzymes and sperm tail movements allow the sperm to penetrate the oocyte layers. Numerous sperm must reach the egg and release acrosomal enzymes before the egg can finally be fertilized. Only one sperm will successfully bind to, penetrate and fertilize the egg, after which the zona hardens so that no other sperm can penetrate the egg (Zaneveld, in Male Infertility Chapter 11, Comhaire (Ed.), Chapman & Hall, London, 1996). Peptide hormones, such as insulin homologs are associated with sperm activation and egg-sperm interaction. For instance, capacitated sperm incubated with relaxin show an increased percentage of progressively motile sperm, increased zona penetration rates, and increased percentage of viable acrosome-reacted sperm (Carrell et al., Endocr. Res. 21:697-707, 1995). Similarity of the ZSLIT3 polypeptide structure with peptide hormones and localization of ZSLIT3 to the testis, prostate and uterus suggests that the ZSLIT3 polypeptides described herein play a role in these and other reproductive processes.

Accordingly, proteins of the present invention can have applications in enhancing fertilization during assisted reproduction in humans and in animals. Such assisted reproduction methods are known in the art and include artificial insemination,

in vitro fertilization, embryo transfer and gamete intrafallopian transfer. Such methods are useful for assisting men and women who have physiological or metabolic disorders preventing natural conception or can be used to enhance *in vitro* fertilization. Such methods are also used in animal breeding programs, such as for livestock breeding and could be used as methods for the creation of transgenic animals. Proteins of the present invention can be combined with sperm, an egg or an egg-sperm mixture prior to fertilization of the egg. In some species, sperm capacitate spontaneously during *in vitro* fertilization procedures, but normally sperm capacitate over an extended period of time both *in vivo* and *in vitro*. It is advantageous to increase sperm activation during such procedures to enhance the likelihood of successful fertilization. The washed sperm or sperm removed from the seminal plasma used in such assisted reproduction methods has been shown to have altered reproductive functions, in particular, reduced motility and zona interaction. To enhance fertilization during assisted reproduction methods sperm is capacitated using exogenously added compounds. Suspension of the sperm in seminal plasma from normal subjects or in a "capacitation media" containing a cocktail of compounds known to activate sperm, such as caffeine, dibutyl cyclic adenosine monophosphate (dbcAMP) or theophylline, have resulted in improved reproductive function of the sperm, in particular, sperm motility and zonae penetration (Park et al., Am. J. Obstet. Gynecol. 158:974-9, 1988; Vandevoort et al., Mol. Repro. Develop. 37:299-304, 1993; Vandevoort and Overstreet, J. Androl. 16:327-33, 1995). The presence of immunoreactive relaxin *in vivo* and in association with cryopreserved semen, was shown to significantly increase sperm motility (Juang et al., Anim. Reprod. Sci. 20:21-9, 1989; Juang et al., Anim. Reprod. Sci. 22:47-53, 1990). Porcine relaxin stimulated sperm motility in cryopreserved human sperm (Colon et al., Fertil. Steril. 46:1133-39, 1986; Lessing et al., Fertil. Steril. 44:406-9, 1985) and preserved ability of washed human sperm to penetrate cervical mucus *in vitro* (Brenner et al., Fertil. Steril. 42:92-6, 1984). Polypeptides of the present invention can used in such methods to enhance viability of cryopreserved sperm, enhance sperm motility and enhance fertilization, particularly in association with methods of assisted reproduction.

In cases where pregnancy is not desired, ZSLIT3 polypeptide or polypeptide fragments may function as germ-cell-specific antigens for use as

components in "immunocontraceptive" or "anti-fertility" vaccines to induce formation of antibodies and/or cell mediated immunity to selectively inhibit a process, or processes, critical to successful reproduction in humans and animals. The use of sperm and testis antigens in the development of immunocontraceptives have been described (O'Hern et al., Biol Reprod. 52:311-39, 1995; Diekman and Herr, Am. J. Reprod. Immunol. 37:111-17, 1997; Zhu and Naz, Proc. Natl. Acad. Sci. USA 94:4704-9,1997). A vaccine based on human chorionic gonadotrophin (HCG) linked to a diphtheria or tetanus carrier was in clinical trials (Talwar et al., Proc. Natl. Acad. Sci. USA 91:8532-36, 1994). A single injection resulted in production of high titer antibodies that persisted for nearly a year in rabbits (Stevens, Am. J. Reprod. Immunol. 29:176-88, 1993). Such methods of immunocontraception using vaccines would include a ZSLIT3 testes-specific protein or fragment thereof. The ZSLIT3 protein or fragments can be conjugated to a carrier protein or peptide, such as tetanus or diphtheria toxoid. An adjuvant, as described above, can be included and the protein or fragment can be noncovalently associated with other molecules to enhance intrinsic immunoreactivity. Methods for administration and methods for determining the number of administrations are known in the art. Such a method might include a number of primary injections over several weeks followed by booster injections as needed to maintain a suitable antibody titer.

Regulation of reproductive function in males and females is controlled in part by feedback inhibition of the hypothalamus and anterior pituitary by blood-borne hormones. Testis proteins, such as activins and inhibins, have been shown to regulate secretion of active molecules including follicle stimulating hormone (FSH) from the pituitary (Ying, Endodcr. Rev. 9:267-93, 1988; Plant et al., Hum. Reprod. 8:41-44,1993). Inhibins, also expressed in the ovaries, have been shown to regulate ovarian functions (Woodruff et al., Endocr. 132:2332-42,1993; Russell et al., J. Reprod. Fertil. 100:115-22, 1994). Relaxin has been shown to be a systemic and local acting hormone regulating follicular and uterine growth (Bagnell et al., J. Reprod. Fertil. 48:127-38, 1993). As such, the polypeptides of the present invention may also have effects on female gametes and reproductive tract. These functions may also be associated with ZSLIT3 polypeptides and may be used to regulate testicular or ovarian functions.

The activity of molecules of the present invention may be measured using a variety of assays that, for example, measure neogenesis or hyperplasia (i.e., proliferation) of cardiac cells based on the potential effects of activity of ZSLIT3. Additional activities likely associated with the polypeptides of the present invention include proliferation of endothelial cells, cardiomyocytes, fibroblasts, skeletal myocytes directly or indirectly through other growth factors; action as a chemotaxic factor for endothelial cells, fibroblasts and/or phagocytic cells; osteogenic factor; and factor for expanding mesenchymal stem cell and precursor populations.

Proliferation can be measured using cultured cardiac cells or *in vivo* by administering molecules of the present invention to the appropriate animal model. Generally, proliferative effects are seen as an increase in cell number, and may include inhibition of apoptosis as well as stimulation of mitogenesis. Cultured cells for use in these assays include cardiac fibroblasts, cardiac myocytes, skeletal myocytes, and human umbilical vein endothelial cells from primary cultures. Suitable established cell lines include: NIH 3T3 fibroblasts (ATCC No. CRL-1658), CHH-1 chum heart cells (ATCC No. CRL-1680), H9c2 rat heart myoblasts (ATCC No. CRL-1446), Shionogi mammary carcinoma cells (Tanaka et al., Proc. Natl. Acad. Sci. 89:8928-8932, 1992), and LNCap.FGC adenocarcinoma cells (ATCC No. CRL-1740.) Assays measuring cell proliferation are well known in the art. For example, assays measuring proliferation include such assays as chemosensitivity to neutral red dye (Cavanaugh et al., Investigational New Drugs 8:347-354, 1990), incorporation of radiolabeled nucleotides (Cook et al., Analytical Biochem. 179:1-7, 1989), incorporation of 5-bromo-2'-deoxyuridine (BrdU) in the DNA of proliferating cells (Porstmann et al., J. Immunol. Methods 82:169-179, 1985), and use of tetrazolium salts (Mosmann, J. Immunol. Methods 65:55-63, 1983; Alley et al., Cancer Res. 48:589-601, 1988; Marshall et al., Growth Reg. 5:69-84, 1995; and Scudiero et al., Cancer Res. 48:4827-4833, 1988).

In vivo assays for evaluating cardiac neogenesis or hyperplasia include treating neonatal and mature rats with the molecules of the present invention. The animals' cardiac function is measured as heart rate, blood pressure, and cardiac output to determine left ventricular function. Post-mortem methods for assessing cardiac decline or improvement include: increased or decreased cardiac weight,

nuclei/cytoplasmic volume, and staining of cardiac histology sections to determine proliferating cell nuclear antigen (PCNA) vs. cytoplasmic actin levels (Quaini et al., Circulation Res. 75:1050-1063, 1994 and Reiss et al., Proc. Natl. Acad. Sci. 93:8630-8635, 1996.)

The present invention also provides methods of studying mammalian cellular metabolism. Such methods of the present invention comprise incubating cells to be studied, for example, human vascular endothelial cells, ± ZSLIT3 polypeptide, monoclonal antibody, agonist or antagonist thereof and observing changes in adipogenesis, gluconeogenesis, glycogenolysis, lipogenesis, glucose uptake, or the like.

Within another aspect of the present invention there is provided a pharmaceutical composition comprising a purified ZSLIT3 polypeptide in combination with a pharmaceutically acceptable vehicle. This pharmaceutical composition may be used to modulate energy balance in mammals or to protect endothelial cells from injury.

With regard to modulating energy balance, ZSLIT3 polypeptides may be used to modulate cellular metabolic reactions. Such metabolic reactions include adipogenesis, gluconeogenesis, glycogenolysis, lipogenesis, lipid metabolism, detoxification and excretion, glucose uptake, protein synthesis, thermogenesis, oxygen utilization and the like. The expression pattern of ZSLIT3 polypeptide indicates expression in major metabolic organs, e.g., liver, pancreas and thyroid, and may have intra- and extra-hepatic and -thyroidal effects on endothelial cell tissues. Such effects involve protection, regeneration, growth and development of liver, pancreas, thyroid or other tissues.

With regard to endothelial cell protection, ZSLIT3 polypeptides may be used in organ preservation, for cryopreservation, for surgical pretreatment to prevent injury due to ischemia and/or inflammation or in like procedures. In this regard, ZSLIT3 polypeptides may find utility in modulating nutrient uptake, as demonstrated, for example, by 2-deoxy-glucose uptake in the brain or the like.

The ZSLIT3 polypeptides may modulate mammalian energy balance. The thyroid expression pattern of ZSLIT3 suggests that ZSLIT3 may exhibit effects on glucose uptake, e.g. through GLUT-1, and thermogenesis (thermoregulation). Among other methods known in the art or described herein, mammalian energy balance may be

evaluated by monitoring one or more of the following metabolic functions: adipogenesis, gluconeogenesis, glycogenolysis, lipogenesis, glucose uptake, protein synthesis, thermogenesis, oxygen utilization or the like. These metabolic functions are monitored by techniques (assays or animal models) known to one of ordinary skill in the art, as is more fully set forth below. For example, the glucoregulatory effects of insulin are predominantly exerted in the liver, skeletal muscle and adipose tissue. Insulin binds to its cellular receptor in these three tissues and initiates tissue-specific actions that result in, for example, the inhibition of glucose production and the stimulation of glucose utilization. In the liver, insulin stimulates glucose uptake and inhibits gluconeogenesis and glycogenolysis. In skeletal muscle and adipose tissue, insulin acts to stimulate the uptake, storage and utilization of glucose.

ZSLIT3 polypeptide is expressed in thyroid but may exhibit extrathyroidal activity in organs that affect metabolic functions. Thus, pharmaceutical compositions of the present invention may be useful in prevention or treatment of pancreatic disorders. For example, ZSLIT3 may be associated with pathological regulation of the expansion of neurocrine and exocrine cells in the pancreas, as evident in IDDM, pancreatic cancer or the like. Pharmaceutical compositions of the present invention may also be involved in prevention or treatment of pancreatic conditions characterized by dysfunction associated with pathological regulation of blood glucose levels, insulin resistance or digestive function.

Art-recognized methods exist for monitoring all of the metabolic functions recited above. Thus, one of ordinary skill in the art is able to evaluate ZSLIT3 polypeptides, fragments, fusion proteins, antibodies, agonists and antagonists for metabolic modulating functions. Exemplary modulating techniques are set forth below.

Adipogenesis, gluconeogenesis and glycogenolysis are interrelated components of mammalian energy balance, which may be evaluated by known techniques using, for example, *ob/ob* mice or *db/db* mice. The *ob/ob* mice are inbred mice that are homozygous for an inactivating mutation at the *ob* (obese) locus. Such *ob/ob* mice are hyperphagic and hypometabolic, and are believed to be deficient in production of circulating OB protein. The *db/db* mice are inbred mice that are

homozygous for an inactivating mutation at the *db* (diabetes) locus. The *db/db* mice display a phenotype similar to that of *ob/ob* mice, except *db/db* mice display a more severe diabetic phenotype. Such *db/db* mice are believed to be resistant to the effects of circulating OB protein. Also, various *in vitro* methods of assessing these parameters are known in the art.

Insulin-stimulated lipogenesis, for example, may be monitored by measuring the incorporation of ^{14}C-acetate into triglyceride (Mackall et al. J. Biol. Chem. 251:6462-6464, 1976) or triglyceride accumulation (Kletzien et al., Mol. Pharmacol. 41:393-398, 1992).

Glucose uptake may be evaluated, for example, in an assay for insulin-stimulated glucose transport. Non-transfected, differentiated L6 myotubes (maintained in the absence of G418) are placed in DMEM containing 1 g/l glucose, 0.5 or 1.0% BSA, 20 mM Hepes, and 2 mM glutamine. After two to five hours of culture, the medium is replaced with fresh, glucose-free DMEM containing 0.5 or 1.0% BSA, 20 mM Hepes, 1 mM pyruvate, and 2 mM glutamine. Appropriate concentrations of insulin or IGF-1, or a dilution series of the test substance, are added, and the cells are incubated for 20-30 minutes. ^{3}H or ^{14}C-labeled deoxyglucose is added to ≈50 1 M final concentration, and the cells are incubated for approximately 10-30 minutes. The cells are then quickly rinsed with cold buffer (e.g. PBS), then lysed with a suitable lysing agent (e.g. 1% SDS or 1 N NaOH). The cell lysate is then evaluated by counting in a scintillation counter. Cell-associated radioactivity is taken as a measure of glucose transport after subtracting non-specific binding as determined by incubating cells in the presence of cytochalasin b, an inhibitor of glucose transport. Other methods include those described by, for example, Manchester et al., Am. J. Physiol. 266 (Endocrinol. Metab. 29):E326-E333, 1994 (insulin-stimulated glucose transport).

Protein synthesis may be evaluated, for example, by comparing precipitation of ^{35}S-methionine-labeled proteins following incubation of the test cells with ^{35}S-methionine and ^{35}S-methionine and a putative modulator of protein synthesis.

Thermogenesis may be evaluated as described by B. Stanley in The Biology of Neuropeptide Y and Related Peptides, W. Colmers and C. Wahlestedt (eds.), Humana Press, Ottawa, 1993, pp. 457-509; C. Billington et al., Am. J. Physiol.

260:R321, 1991; N. Zarjevski et al., Endocrinology 133:1753, 1993; C. Billington et al., Am. J. Physiol. 266:R1765, 1994; Heller et al., Am. J. Physiol. 252(4 Pt 2): R661-7, 1987; and Heller et al., Am. J. Physiol. 245(3): R321-8, 1983. Also, metabolic rate, which may be measured by a variety of techniques, is an indirect measurement of thermogenesis.

Oxygen utilization may be evaluated as described by Heller et al., Pflugers Arch 369(1): 55-9, 1977. This method also involved an analysis of hypothalmic temperature and metabolic heat production. Oxygen utilization and thermoregulation have also been evaluated in humans as described by Haskell et al., J. Appl. Physiol. 51(4): 948-54, 1981.

The ZSLIT3 polypeptides of the present invention may act in the neuroendocrine/exocrine cell fate decision pathway and is therefore capable of regulating the expansion of neuroendocrine and exocrine cells in the pancreas. One such regulatory use is that of islet cell regeneration. Also, it has been hypothesized that the autoimmunity that triggers IDDM starts in utero, and ZSLIT3 polypeptide is a developmental gene involved in cell partitioning. Assays and animal models are known in the art for monitoring the exocrine/neuroendocrine cell lineage decision, for observing pancreatic cell balance and for evaluating ZSLIT3 polypeptide, fragment, fusion protein, antibody, agonist or antagonist in the prevention or treatment of the conditions set forth above.

The cardiac activity of molecules of the present invention may be measured using a Langendorff assay. This preferred assay measures *ex vivo* cardiac function for an experimental animal, and is well known in the art. Experimental animals are, for example but not limited to, rats, rabbits and guinea pigs. Chronic effects on heart tissue can be measured after treating a test animal with ZSLIT3 polypeptide for 1 to 7 days, or longer. Control animals will have only received buffer. After treatment, the heart is removed and perfused retrograde through the aorta. During perfusion, several physiologic parameters are measured: coronary blood flow per time, left ventricular (LV) pressures, and heart rate. These perameters directly reflect cardiac function. Changes in these parameters, as measured by the Langendorff assay, following *in vivo* treatment with ZSLIT3 polypeptide relative to control animals

indicates a chronic effect of the polypeptide on heart function. Moreover, the Langendorff assay can also be employed to measure the acute effects of ZSLIT3 polypeptide on heart. In such application, hearts from untreated animals are used and ZSLIT3 polypeptide is added to the perfusate in the assay. The parameters assessed above are measured and compared with the results from control hearts where ZSLIT3 polypeptide was omitted from the perfusate. Differences in heart rate, change in pressure per time, and/or coronary blood flow indicate an acute effect of the molecules of the present invention on heart function.

The molecules of the present invention may be useful for proliferation of cardiac tissue cells, such as cardiac myocytes or myoblasts; skeletal myocytes or myoblasts and smooth muscle cells; chrondrocytes; endothelial cells; adipocytes and osteoblasts *in vitro*. For example, molecules of the present invention are useful as components of defined cell culture media, and may be used alone or in combination with other cytokines and hormones to replace serum that is commonly used in cell culture. Molecules of the present invention are particularly useful in specifically promoting the growth and/or development of myocytes in culture, and may also prove useful in the study of cardiac myocyte hyperplasia and regeneration.

The polypeptides, nucleic acids and/or antibodies of the present invention may be used in treatment of disorders associated with myocardial infarction, congestive heart failure, hypertrophic cardiomyopathy and dilated cardiomyopathy. Molecules of the present invention may also be useful for limiting infarct size following a heart attack, aiding in recovery after heart transplantation, promoting angiogenesis and wound healing following angioplasty or endarterectomy, to develop coronary collateral circulation, for revascularization in the eye, for complications related to poor circulation such as diabetic foot ulcers, for stroke, following coronary reperfusion using pharmacologic methods, and other indications where angiogenesis is of benefit. Molecules of the present invention may be useful for improving cardiac function, either by inducing cardiac myocyte neogenesis and/or hyperplasia, by inducing coronary collateral development, or by inducing remodeling of necrotic myocardial area. Other therapeutic uses for the present invention include induction of skeletal muscle

neogenesis and/or hyperplasia, kidney regeneration and/or for treatment of systemic and pulmonary hypertension.

ZSLIT3 induced coronary collateral development is measured in rabbits, dogs or pigs using models of chronic coronary occlusion (Landau et al., Amer. Heart J. 29:924-931, 1995; Sellke et al., Surgery 120(2):182-188, 1996; and Lazarous et al., 1996, ibid.) ZSLIT3 efficacy for treating stroke is tested *in vivo*, in rats, utilizing bilateral carotid artery occlusion and measuring histological changes, as well as maze performance (Gage et al., Neurobiol. Aging 9:645-655, 1988). ZSLIT3 efficacy in hypertension is tested *in vivo* utilizing spontaneously hypertensive rats (SHR) for systemic hypertension (Marche et al., Clin. Exp. Pharmacol. Physiol. Suppl. 1:S114-116, 1995).

ZSLIT3 polypeptides can also be used to prepare antibodies that bind to ZSLIT3 epitopes, peptides or polypeptides. The ZSLIT3 polypeptide or a fragment thereof serves as an antigen (immunogen) to inoculate an animal and elicit an immune response. One of skill in the art would recognize that antigenic, epitope-bearing polypeptides contain a sequence of at least 6, preferably at least 9, and more preferably at least 15 to about 30 contiguous amino acid residues of a ZSLIT3 polypeptide (e.g., SEQ ID NO:2). Polypeptides comprising a larger portion of a ZSLIT3 polypeptide, i.e., from 30 to 10 residues up to the entire length of the amino acid sequence are included. Antigens or immunogenic epitopes can also include attached tags, adjuvants and carriers, as described herein. Suitable antigens include the ZSLIT3 polypeptide encoded by SEQ ID NO:2 from amino acid number 24 (Cys) to amino acid number 673 (Ile) or a contiguous 9 to 649 amino acid fragment thereof. Other suitable antigens include the N-terminal LRR flanking domain, LRR domain, LRR motifs LRR-1-10, middle region, C-terminal LRR flanking domain, EGF domain, C-terminal region and other domains and motifs as described herein. Preferred peptides to use as antigens are hydrophilic peptides such as those predicted by one of skill in the art from a hydrophobicity plot. ZSLIT3 hydrophilic peptides include peptides comprising amino acid sequences selected from the group consisting of: (1) amino acid number 123 (Leu) to amino acid number 128 (Arg) of SEQ ID NO:2; (2) amino acid number 156 (Gln) to amino acid number 161 (Arg) of SEQ ID NO:2; (3) amino acid number 225 (Asp) to

amino acid number 230 (Arg) of SEQ ID NO:2; (4) amino acid number 322 (Ser) to amino acid number 327 (Arg) of SEQ ID NO:2; and (5) amino acid number 502 (Ser) to amino acid number 507 (Arg) of SEQ ID NO:2. Moreover, ZSLIT3 antigenic epitopes as predicted by a Jameson-Wolf plot, e.g., using DNASTAR Protean program (DNASTAR, Inc., Madison, WI) serve as suitable antigens. Antibodies from an immune response generated by inoculation of an animal with these antigens can be isolated and purified as described herein. Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, Current Protocols in Immunology, Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, 1989; and Hurrell, J. G. R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, FL, 1982.

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with a ZSLIT3 polypeptide or a fragment thereof. The immunogenicity of a ZSLIT3 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of ZSLIT3 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as $F(ab')_2$ and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting non-human

CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced. Moreover, human antibodies can be produced in transgenic, non-human animals that have been engineered to contain human immunoglobulin genes as disclosed in WIPO Publication WO 98/24893. It is preferred that the endogenous immunoglobulin genes in these animals be inactivated or eliminated, such as by homologous recombination.

Antibodies are considered to be specifically binding if: 1) they exhibit a threshold level of binding activity, and 2) they do not significantly cross-react with related polypeptide molecules. A threshold level of binding is determined if anti-ZSLIT3 antibodies herein bind to a ZSLIT3 polypeptide, peptide or epitope with an affinity at least 10-fold greater than the binding affinity to control (non-ZSLIT3) polypeptide. It is preferred that the antibodies exhibit a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis (Scatchard, G., Ann. NY Acad. Sci. 51: 660-672, 1949).

Whether anti-ZSLIT3 antibodies do not significantly cross-react with related polypeptide molecules is shown, for example, by the antibody detecting ZSLIT3 polypeptide but not known related polypeptides using a standard Western blot analysis (Ausubel et al., ibid.). Examples of known related polypeptides are those disclosed in the prior art, such as known orthologs, and paralogs, and similar known members of a protein family, Screening can also be done using non-human ZSLIT3, and ZSLIT3 mutant polypeptides. Moreover, antibodies can be "screened against" known related polypeptides, to isolate a population that specifically binds to the ZSLIT3 polypeptides. For example, antibodies raised to ZSLIT3 are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to ZSLIT3 will flow through the matrix under

the proper buffer conditions. Screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to known closely related polypeptides (Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988; Current Protocols in Immunology, Cooligan, et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art. See, Fundamental Immunology, Paul (eds.), Raven Press, 1993; Getzoff et al., *Adv. in Immunol.* 43: 1-98, 1988; Monoclonal Antibodies: Principles and Practice, Goding, J.W. (eds.), Academic Press Ltd., 1996; Benjamin et al., Ann. Rev. Immunol. 2: 67-101, 1984. Specifically binding anti-ZSLIT3 antibodies can be detected by a number of methods in the art, and disclosed below.

A variety of assays known to those skilled in the art can be utilized to detect antibodies which bind to ZSLIT3 proteins or polypeptides. Exemplary assays are described in detail in Antibodies: A Laboratory Manual, Harlow and Lane (Eds.), Cold Spring Harbor Laboratory Press, 1988. Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant ZSLIT3 protein or polypeptide.

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to ZSLIT3 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled ZSLIT3 protein or peptide). Genes encoding polypeptides having potential ZSLIT3 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli.* Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al.,

US Patent NO. 4,946,778; Ladner et al., US Patent NO. 5,403,484 and Ladner et al., US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the ZSLIT3 sequences disclosed herein to identify proteins which bind to ZSLIT3. These "binding polypeptides" which interact with ZSLIT3 polypeptides can be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding polypeptides can also be used in analytical methods such as for screening expression libraries and neutralizing activity, e.g., for blocking interaction between ligand and receptor, or viral binding to a receptor. The binding polypeptides can also be used for diagnostic assays for determining circulating levels of ZSLIT3 polypeptides; for detecting or quantitating soluble ZSLIT3 polypeptides as marker of underlying pathology or disease. These binding polypeptides can also act as ZSLIT3 "antagonists" to block ZSLIT3 binding and signal transduction *in vitro* and *in vivo*. These anti-ZSLIT3 binding polypeptides would be useful for inhibiting ZSLIT3 activity or protein-binding.

Antibodies to ZSLIT3 may be used for tagging cells that express ZSLIT3; for isolating ZSLIT3 by affinity purification; for diagnostic assays for determining circulating levels of ZSLIT3 polypeptides; for detecting or quantitating soluble ZSLIT3 as marker of underlying pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block ZSLIT3 activity *in vitro* and *in vivo*. Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Moreover, antibodies to ZSLIT3 or fragments thereof may be used *in vitro* to detect denatured

ZSLIT3 or fragments thereof in assays, for example, Western Blots or other assays known in the art.

In the alternative, the ZSLIT3 polypeptide itself can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. These ZSLIT3 polypeptide fusions would be useful for identifying, monitoring or activating ZSLIT3 activity or used to specifically kill cells in which SLIT3 receptor is over-expressed, for example in ZSLIT3 receptor-expressing cancers.

Genes encoding polypeptides having potential ZSLIT3 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli.* Nucleotide sequences encoding the binding polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al., US Patent NO. 4,946,778; Ladner et al., US Patent NO. 5,403,484 and Ladner et al., US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the ZSLIT3 sequences disclosed herein to identify proteins which bind to ZSLIT3. These "binding polypeptides" which interact with ZSLIT3 polypeptides can be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding polypeptides can also be used in analytical methods such as for screening expression libraries and neutralizing activity. The binding polypeptides can also be used for diagnostic assays for determining circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying pathology or disease. These

binding polypeptides can also act as ZSLIT3 "antagonists" to block ZSLIT3 binding and signal transduction *in vitro* and *in vivo*. These anti-ZSLIT3 binding polypeptides would be useful for inhibiting ZSLIT3 binding.

ZSLIT3 polypeptides and polynucleotides may be used within diagnostic systems. Antibodies or other agents that specifically bind to ZSLIT3 may be used to detect the presence of circulating ligand or receptor polypeptides. Such detection methods are well known in the art and include, for example, enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay. Immunohistochemically labeled ZSLIT3 antibodies can be used to detect ZSLIT3 receptor and/or ligands in tissue samples and identify ZSLIT3 receptors. ZSLIT3 levels can also be monitored by such methods as RT-PCR, where ZSLIT3 mRNA can be detected and quantified. The information derived from such detection methods would provide insight into the significance of ZSLIT3 polypeptides in various diseases and biological processes, and as such would serve as diagnostic tools for diseases for which altered levels of ZSLIT3 are significant.

Nucleic acid molecules disclosed herein can be used to detect the expression of a ZSLIT3 gene in a biological sample. Such probe molecules include double-stranded nucleic acid molecules comprising the nucleotide sequences of SEQ ID NO:1 or SEQ ID NO:3, or fragments thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequences of SEQ ID NO:1 or SEQ ID NO:3, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like. For example, suitable probes include nucleic acid molecules that bind with a portion of a ZSLIT3 domain or motif disclosed herein, such as the ZSLIT3 slit protein domain. Other probes include those to the N-terminal LRR flanking domain, LRR domain, LRR motifs LRR-1-10, middle region, C-terminal LRR flanking domain, EGF domain, C-terminal region and other domains and motifs as described herein. In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target ZSLIT3 RNA species. After separating unbound probe from hybridized molecules, the level and length of the hybrid is detected. Well-established hybridization methods of RNA detection include

northern analysis and dot/slot blot hybridization, see, for example, Ausubel ibid. and Wu *et* al. (eds.), "Analysis of Gene Expression at the RNA Level," in Methods in Gene Biotechnology, pages 225-239 (CRC Press, Inc. 1997), and methods described herein. Nucleic acid probes can be detectably labeled with radioisotopes such as ^{32}P or ^{35}S. Alternatively, ZSLIT3 RNA can be detected with a nonradioactive hybridization method (see, for example, Isaac (ed.), Protocols for Nucleic Acid Analysis by Nonradioactive Probes, Humana Press, Inc., 1993). Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative non-radioactive moieties include biotin, fluorescein, and digoxigenin.

ZSLIT3 oligonucleotide probes are also useful for *in vivo* diagnosis. For example, ^{18}F-labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography (Tavitian et al., Nature Medicine 4:467, 1998). Moreover, numerous diagnostic procedures take advantage of the polymerase chain reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known (see, generally, Mathew (ed.), Protocols in Human Molecular Genetics (Humana Press, Inc. 1991), White (ed.), PCR Protocols: Current Methods and Applications (Humana Press, Inc. 1993), Cotter (ed.), Molecular Diagnosis of Cancer (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), Tumor Marker Protocols (Humana Press, Inc. 1998), Lo (ed.), Clinical Applications of PCR (Humana Press, Inc. 1998), and Meltzer (ed.), PCR in Bioanalysis (Humana Press, Inc. 1998)). PCR primers can be designed to amplify a sequence encoding a full-length or partial ZSLIT3 polynucleotide, or a particular ZSLIT3 domain or motif, such as the ZSLIT3 LRR or EGF domains as disclosed herein.

One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample, reverse transcribed to cDNA, and the cDNA is incubated with ZSLIT3 primers (see, for example, Wu et al. (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in Methods in Gene Biotechnology, CRC Press, Inc., pages 15-28, 1997). PCR is then performed and the products are analyzed using standard techniques. For example, RNA is isolated from biological sample using, for example, the guanidinium-isothiocyanate cell lysis procedure described herein. Alternatively, a solid-phase technique can be

used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random oligonucleotides, short homopolymers of dT, or ZSLIT3 anti-sense oligomers. Oligo-dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. ZSLIT3 sequences are amplified by the polymerase chain reaction using two flanking oligonucleotide primers. PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can be transferred to a membrane, hybridized with a detectably-labeled ZSLIT3 probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay. Another approach is to use real time quantitative PCR (Perkin-Elmer Cetus, Norwalk, Ct.). A fluorogenic probe, consisting of an oligonucleotide with both a reporter and a quencher dye attached, anneals specifically between the forward and reverse primers. Using the 5' endonuclease activity of Taq DNA polymerase, the reporter dye is separated from the quencher dye and a sequence-specific signal is generated and increases as amplification increases. The fluorescence intensity can be continuously monitored and quantified during the PCR reaction.

Another approach for detection of ZSLIT3 expression is cycling probe technology (CPT), in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNase H, and the presence of cleaved chimeric probe is detected (see, for example, Beggs et al., J. Clin. Microbiol. 34:2985, 1996 and Bekkaoui et al., Biotechniques 20:240, 1996). Alternative methods for detection of ZSLIT3 sequences can utilize approaches such as nucleic acid sequence-based amplification (NASBA), cooperative amplification of templates by cross-hybridization (CATCH), and the ligase chain reaction (LCR) (see, for example, Marshall et al., U.S. Patent No. 5,686,272 (1997), Dyer et al., J. Virol. Methods 60:161, 1996; Ehricht et al., Eur. J. Biochem. 243:358, 1997 and Chadwick et al., J. Virol. Methods 70:59, 1998). Other standard methods are known to those of skill in the art.

ZSLIT3 probes and primers can also be used to detect and to localize ZSLIT3 gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), In Situ Hybridization Protocols, Humana Press, Inc., 1994; Wu et al. (eds.), "Analysis of Cellular DNA or Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in Methods in Gene Biotechnology, CRC Press, Inc., pages 259-278, 1997 and Wu et al. (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in Methods in Gene Biotechnology, CRC Press, Inc., pages 279-289, 1997). Various additional diagnostic approaches are well-known to those of skill in the art (see, for example, Mathew (ed.), Protocols in Human Molecular Genetics Humana Press, Inc., 1991; Coleman and Tsongalis, Molecular Diagnostics, Humana Press, Inc., 1996 and Elles, Molecular Diagnosis of Genetic Diseases, Humana Press, Inc., 1996).

The ZSLIT3 polynucleotides and/or polypeptides disclosed herein can be useful as therapeutics, wherein ZSLIT3 agonists and antagonists could modulate one or more biological processes in cells, tissues and/or biological fluids. ZSLIT3 antagonists provided by the invention, bind to ZSLIT3 polypeptides or, alternatively, to a receptor to which ZSLIT3 polypeptides bind, thereby inhibiting or eliminating the function of ZSLIT3. Such ZSLIT3 antagonists would include antibodies; oligonucleotides which bind either to the ZSLIT3 polypeptide or to its ligand; natural or synthetic analogs of ZSLIT3 ligands which retain the ability to bind the receptor but do not result in either ligand or receptor signaling. Such analogs could be peptides or peptide-like compounds. Natural or synthetic small molecules which bind to ZSLIT3 polypeptides and prevent signaling are also contemplated as antagonists. As such, ZSLIT3 antagonists would be useful as therapeutics for treating certain disorders where blocking signal from either a ZSLIT3 receptor or ligand would be beneficial.

The invention also provides nucleic acid-based therapeutic treatment. If a mammal lacks or has a mutated ZSLIT3 gene, the ZSLIT3 gene can be introduced into the cells of the mammal. Using such methods, cells altered to express the nerve growth factor neurotrophin-3 (NT-3) were grafted to a rat model for spinal injury and stimulated axon regrowth at the lesion site and the rats thus treated recovered some

ability to walk (Grill et al., J. Neuroscience 17:5560-72, 1997). In one embodiment, a gene encoding a ZSLIT3 polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt et al., Molec. Cell. Neurosci. 2:320-30, 1991), an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al. (J. Clin. Invest. 90:626-30, 1992), and a defective adeno-associated virus vector (Samulski et al., J. Virol. 61:3096-101, 1987; Samulski et al., J. Virol. 63:3822-8, 1989).

In another embodiment, the gene can be introduced in a retroviral vector, *e.g.*, as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al., Cell 33:153, 1983; Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., J. Virol. 62:1120, 1988; Temin et al., U.S. Patent No. 5,124,263; Dougherty et al., WIPO Publication WO 95/07358; and Kuo et al., Blood 82:845-52, 1993.

Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7, 1987; and Mackey et al., Proc. Natl. Acad. Sci. USA 85:8027-31, 1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cells represents one area of benefit. It is clear that directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides, e.g., hormones or

neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is possible to remove the cells from the body and introduce the vector as a naked DNA plasmid and then re-implant the transformed cells into the body. Naked DNA vector for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter (see, for example, Wu et al., J. Biol. Chem. 267:963-7, 1992; Wu et al., J. Biol. Chem. 263:14621-4, 1988).

Another aspect of the present invention involves antisense polynucleotide compositions that are complementary to a segment of the polynucleotide set forth in SEQ ID NO:1. Such synthetic antisense oligonucleotides are designed to bind to mRNA encoding ZSLIT3 polypeptides and to inhibit translation of such mRNA. Such antisense oligonucleotides are used to inhibit expression of ZSLIT3 polypeptide-encoding genes in cell culture or in a subject.

The present invention also provides reagents which will find use in diagnostic applications. For example, the ZSLIT3 gene, a probe comprising ZSLIT3 DNA or RNA or a subsequence thereof can be used to determine if the ZSLIT3 gene is present on a human chromosome, such as chromosome 16, or if a mutation has occurred. Based on annotation of a fragment of human genomic DNA containing the ZSLIT3 genomic DNA (Genbank Accession No. AC006208), ZSLIT3 is located at the 16q12 region of chromosome 16. Detectable chromosomal aberrations at the ZSLIT3 gene locus include, but are not limited to, aneuploidy, gene copy number changes, translocations, insertions, deletions, loss of heterogeneity, restriction site changes and rearrangements. Such aberrations can be detected using polynucleotides of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, and other genetic linkage analysis techniques known in the art (Sambrook et al., ibid.; Ausubel et. al., ibid.; Marian, Chest 108:255-65, 1995).

The precise knowledge of a gene's position can be useful for a number of purposes, including: 1) determining if a sequence is part of an existing contig and

obtaining additional surrounding genetic sequences in various forms, such as YACs, BACs or cDNA clones; 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal region; and 3) cross-referencing model organisms, such as mouse, which may aid in determining what function a particular gene might have.

The ZSLIT3 gene is located on chromosome 16. Several genes of known function map to chromosome 16. For example, the interleukin 4 (IL-4) cytokine receptor alpha-subunit, a member of the hematopoietin receptor family, maps to 16p12.1-p11.2. Moreover, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with defects in IL-4 receptor, such as those that are implicated in some allergic inflammatory disorders and asthma (Deichman, K.A. et al., Exp. Allergy 28:151-155; 1998; Mitsuyasu, H. et al., Nature Genet. 19:119-120, 1998). In addition, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with inflammatory bowel disease, where a susceptibility marker maps to 16p12-q13 (Cho, J.H. et al, Proc. Nat. Acad. Sci. 95:7502-7507, 1998). Further, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with hemoglobin loci located at 16pter-p13.3, and particularly hemoglobin-alpha defects associated with alpha-thalassemia syndromes, such as hydrops fetalis (for review, see Chui, M.P., and Waye, J.S. Blood 91:2213-2222, 1998). Moreover, amongst other genetic loci, those for Wilms tumor, type III (16q), Rubenstein-Taybi syndrome (16p13.3), severe infantile polycystic kidney disease (16p13.3), Townes-Brocks Syndrome (16q12.1), Tuberous sclerosis 2 (16p13.3), Batten disease (ceroid lipofuscinosis, neuronal 3, juvenile) (16p12.1) all manifest themselves in human disease states as well as map to this region of the human genome. See the Online Mendellian Inheritance of Man (OMIM™, National Center for Biotechnology Information, National Library of Medicine. Bethesda, MD) gene map, and references therein, for this region of chromosome 16 on a publicly available WWW server (http://www3.ncbi.nlm.nih.gov/htbin-post/Omim/getmap?chromosome=16q12). All of these serve as possible candidate genes for an inheritable disease which show linkage to the same chromosomal region as the ZSLIT3 gene.

Moreover, ZSLIT3 polynucleotide probes can be used to detect gross abnormalities or genotypes such as chromosome 16 translocations, loss, and gain. For example, Townes-Brocks Syndrome (TBS) (16q21.1) mentioned above, results in a reciprocal translocation between chromosomes 5 and 16 with a breakpoint at 16q12.1, within the same chromosomal locus as ZLIT3 (Friedman, PA et al., Am. J. Hum. Genet. 41(S):A60, 1987; and Serville, F et al., Genet. Counseling 4:109-112, 1993). Moreover, LOH at 16q (16q12-q13) occurs in 70% of familial cylindroma neoplasms (Biggs, PJ et al., Oncogene 12:1375-1377, 1996). In addition, chromosome 16 trisomy results in an increase of mutant APRT expression (16q24.3) associated with disease; and a chromosomal breakpoint exists at 16q12-22. One of skill in the art would recognize that gross chromosomal abnormalities in and around the 16q12 gene locus, wherein the ZSLIT3 gene is located, are present in human disease, and therefore ZSLIT3 probes could be used specifically to detect such abnormalities in addition to detection of mutations within the ZSLIT gene itself. A diagnostic could assist physicians in determining the type of genetic disease and appropriate associated therapy, or assistance in genetic counseling. As such, the inventive anti-ZSLIT3 antibodies, polynucleotides, and polypeptides can be used for the detection of ZSLIT3 polypeptide, mRNA or anti-ZSLIT3 antibodies, thus serving as markers and be directly used for detecting or diagnosing diseases or cancers, as described herein, using methods known in the art and described herein. Further, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with chromosome 16 deletions, translocations and aneuploidy associated with human diseases, such as those associated with the fragile site at 16q22 which are expected to be involved in chromosome rearrangements in cancers and other disease states. Chromosome loss of suppressor genes is often associated with malignancy or other stages in tumor progression. Similarly, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with chromosome 16 trisomy and chromosome loss associated with human diseases such as Wilms Tumor (above; 16q loss), and polycystic kidney disease (above), Tuberous sclerosis 2 (above).

Similarly, defects in the ZSLIT3 gene itself may result in a heritable human disease state. Such defects may result in abnormal neuronal development,

neurologic disease, retardation, organ failure or other disease states. Molecules of the present invention, such as the polypeptides, antagonists, agonists, polynucleotides and antibodies of the present invention would aid in the detection, diagnosis prevention, and treatment associated with a ZSLIT3 genetic defect.

In addition, ZSLIT3 polynucleotide probes can be used to detect allelic differences between diseased or non-diseased individuals at the ZSLIT3 chromosomal locus. As such, the ZSLIT3 sequences can be used not only as diagnostics for disease, but as as diagnostics in forensic DNA profiling.

Similarly, defects in the ZSLIT3 gene itself may result in a heritable human disease state. The ZSLIT3 gene (16q12) is located near genes involved in human disease, and one of skill in the art would appreciate that defects in 16q12 are known to cause disease states in humans. Thus, similarly, defects in ZSLIT3 can cause a disease state or susceptibility to disease. As, ZSLIT3 is a slit protein in a chromosomal hot spot for translocations and LOH involved in genetic diseases and is shown to be expressed in intestinal, brain, breast and osteogenic sarcoma cancer cells, the molecules of the present invention could also be directly involved in cancer formation or metastasis. As the ZSLIT3 gene is located at the 16q12 region ZSLIT3, polynucleotide probes can be used to detect chromosome 16q12 loss, trisomy, duplication or translocation associated with human diseases, such as brain tumors, breast, intestinal and osteogenic tumors and diseased brain, ovary or testis cancers, or diseases. Moreover, molecules of the present invention, such as the polypeptides, antagonists, agonists, polynucleotides and antibodies of the present invention would aid in the detection, diagnosis prevention, and treatment associated with a ZSLIT3 genetic defect.

A diagnostic could assist physicians in determining the type of disease and appropriate associated therapy, or assistance in genetic counseling. As such, the inventive anti-ZSLIT3 antibodies, polynucleotides, and polypeptides can be used for the detection of ZSLIT3 polypeptide, mRNA or anti-ZSLIT3 antibodies, thus serving as markers and be directly used for detecting or genetic diseases or cancers, as described herein, using methods known in the art and described herein. Further, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with

chromosome 16q12 deletions and translocations associated with human diseases, other translocations involved with malignant progression of tumors or other 16q12 mutations, which are expected to be involved in chromosome rearrangements in malignancy; or in other cancers, or in spontaneous abortion. Similarly, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with chromosome 16q12 trisomy and chromosome loss associated with human diseases. Thus, ZSLIT3 polynucleotide probes can be used to detect abnormalities or genotypes associated with these defects.

As discussed above, defects in the ZSLIT3 gene itself may result in a heritable human disease state. Molecules of the present invention, such as the polypeptides, antagonists, agonists, polynucleotides and antibodies of the present invention would aid in the detection, diagnosis prevention, and treatment associated with a ZSLIT3 genetic defect. In addition, ZSLIT3 polynucleotide probes can be used to detect allelic differences between diseased or non-diseased individuals at the ZSLIT3 chromosomal locus. As such, the ZSLIT3 sequences can be used as diagnostics in forensic DNA profiling.

In general, the diagnostic methods used in genetic linkage analysis, to detect a genetic abnormality or aberration in a patient, are known in the art. Analytical probes will be generally at least 20 nt in length, although somewhat shorter probes can be used (e.g., 14-17 nt). PCR primers are at least 5 nt in length, preferably 15 or more, more preferably 20-30 nt. For gross analysis of genes, or chromosomal DNA, a ZSLIT3 polynucleotide probe may comprise an entire exon or more. Exons are readily determined by one of skill in the art by comparing ZSLIT3 sequences (SEQ ID NO:1) with the human genomic DNA for ZSLIT3 (Genbank Accession No. AC007226). In general, the diagnostic methods used in genetic linkage analysis, to detect a genetic abnormality or aberration in a patient, are known in the art. Most diagnostic methods comprise the steps of (a) obtaining a genetic sample from a potentially diseased patient, diseased patient or potential non-diseased carrier of a recessive disease allele; (b) producing a first reaction product by incubating the genetic sample with a ZSLIT3 polynucleotide probe wherein the polynucleotide will hybridize to complementary polynucleotide sequence, such as in RFLP analysis or by incubating the genetic sample

with sense and antisense primers in a PCR reaction under appropriate PCR reaction conditions; (iii) Visualizing the first reaction product by gel electrophoresis and/or other known method such as visualizing the first reaction product with a ZSLIT3 polynucleotide probe wherein the polynucleotide will hybridize to the complementary polynucleotide sequence of the first reaction; and (iv) comparing the visualized first reaction product to a second control reaction product of a genetic sample from wild type patient. A difference between the first reaction product and the control reaction product is indicative of a genetic abnormality in the diseased or potentially diseased patient, or the presence of a heterozygous recessive carrier phenotype for a non-diseased patient, or the presence of a genetic defect in a tumor from a diseased patient, or the presence of a genetic abnormality in a fetus or pre-implantation embryo. For example, a difference in restriction fragment pattern, length of PCR products, length of repetitive sequences at the ZSLIT3 genetic locus, and the like, are indicative of a genetic abnormality, genetic aberration, or allelic difference in comparison to the normal wild type control. Controls can be from unaffected family members, or unrelated individuals, depending on the test and availability of samples. Genetic samples for use within the present invention include genomic DNA, mRNA, and cDNA isolated form any tissue or other biological sample from a patient, such as but not limited to, blood, saliva, semen, embryonic cells, amniotic fluid, and the like. The polynucleotide probe or primer can be RNA or DNA, and will comprise a portion of SEQ ID NO:1, the complement of SEQ ID NO:1, or an RNA equivalent thereof. Such methods of showing genetic linkage analysis to human disease phenotypes are well known in the art. For reference to PCR based methods in diagnostics see see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.), *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 1998)).

Aberrations associated with the ZSLIT3 locus can be detected using nucleic acid molecules of the present invention by employing standard methods for direct mutation analysis, such as restriction fragment length polymorphism analysis,

short tandem repeat analysis employing PCR techniques, amplification-refractory mutation system analysis, single-strand conformation polymorphism detection, RNase cleavage methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis, and other genetic analysis techniques known in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest 108*:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Human Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), *Genome Analysis, Vol. 2: Detecting Genes* (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and Ward, "Molecular Diagnostic Testing," in *Principles of Molecular Medicine*, pages 83-88 (Humana Press, Inc. 1998)). Direct analysis of an ZSLIT3 gene for a mutation can be performed using a subject's genomic DNA. Methods for amplifying genomic DNA, obtained for example from peripheral blood lymphocytes, are well-known to those of skill in the art (see, for example, Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics*, at pages 7.1.6 to 7.1.7 (John Wiley & Sons 1998)).

Mice engineered to express the ZSLIT3 gene, referred to as "transgenic mice," and mice that exhibit a complete absence of ZSLIT3 gene function, referred to as "knockout mice," may also be generated (Snouwaert et al., Science 257:1083, 1992; Lowell et al., Nature 366:740-42, 1993; Capecchi, M.R., Science 244: 1288-1292, 1989; Palmiter, R.D. et al. Annu Rev Genet. 20: 465-499, 1986). For example, transgenic mice that over-express ZSLIT3, either ubiquitously or under a tissue-specific or tissue-restricted promoter can be used to ask whether over-expression causes a phenotype. For example, over-expression of a wild-type ZSLIT3 polypeptide, polypeptide fragment or a mutant thereof may alter normal cellular processes, resulting in a phenotype that identifies a tissue in which ZSLIT3 expression is functionally relevant and may indicate a therapeutic target for the ZSLIT3, its agonists or antagonists. For example, a preferred transgenic mouse to engineer is one that over-expresses the mature human ZSLIT3 polypeptide (residue 24 (Cys) to residue 673 (Ile) of SEQ ID NO:2). Moreover, such over-expression may result in a phenotype that

shows similarity with human diseases. Similarly, knockout ZSLIT3 mice can be used to determine where ZSLIT3 is absolutely required *in vivo*. The phenotype of knockout mice is predictive of the *in vivo* effects of that a ZSLIT3 antagonist, such as those described herein, may have. The murine ZSLIT3 mRNA, and cDNA can be isolated and used to isolate mouse ZSLIT3 genomic DNA, which are subsequently used to generate knockout mice. These transgenic and knockout mice may be employed to study the ZSLIT3 gene and the protein encoded thereby in an *in vivo* system, and can be used as *in vivo* models for corresponding human or animal diseases (such as those in commercially viable animal populations). The mouse models of the present invention are particularly relevant as tumor models for the study of cancer biology and progression. Such models are useful in the development and efficacy of therapeutic molecules used in human cancers. Because increases in ZSLIT3 expression, as well as decreases in ZSLIT3 expression are associated with specific human cancers, both transgenic mice and knockout mice would serve as useful animal models for cancer. Moreover, in a preferred embodiment, ZSLIT3 transgenic mouse can serve as an animal model for specific tumors, particularly glioblastoma, intestinal and breast cancers and osteogenic sarcomas. Moreover, transgenic mice expression of ZSLIT3 antisense polynucleotides or ribozymes directed against ZSLIT3, described herein, can be used analogously to transgenic mice described above.

For pharmaceutical use, the proteins of the present invention are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a ZSLIT3 polypeptide in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington: The Science and Practice of Pharmacy, Gennaro, ed., Mack Publishing Co., Easton, PA, 19th ed., 1995. Determination of dose is within the level of ordinary skill in the art.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1

Identification and Cloning of ZSLIT3

Scanning of a translated human cDNA database resulted in identification of the expressed tag (EST) sequence which was used to identify a human full length cDNA sequence. The cDNA sequence has homology to the slit family of proteins and been designated ZSLIT3.

The entire insert of the plasmid containing the EST sequence was sequenced and found to have the initiation Met and contain most of the 5' sequence. A human genomic sequence was identified corresponding to the zslit3 gene which completed the 3' end of the cDNA thus providing a full length coding sequence for ZSLIT3. The full length cDNA sequence is shown in SEQ ID NO:1 and its corresponding polypeptide sequence is shown in SEQ ID NO:2.

Example 2

Tissue Distribution

Northern blot analysis was performed using Human Multiple Tissue Northern™ Blots (MTN I, MTN II, and MTN III) (Clontech). A SAOS2 marathon library (a human primary osteogenic sarcoma cell line) was used in a PCR reaction using oligos ZC23,641 (SEQ ID NO:5), and ZC23,642 (SEQ ID NO:6) as primers. PCR conditions were as follows: 94°C for 1 minute; then 30 cycles of 94°C, 20 seconds; 61°C, 30 seconds; 72°C, 30 seconds; then ended with a final extension at 72°C for 5 minutes. A sample of the PCR reaction product was run on a 4% agarose gel. A band of the expected size of 116 bp was seen. The 116 bp PCR fragment, was gel purified using a commercially available kit (QiaQuick Gel Extraction Kit; Qiagen) and re-amplified according to conditions above except for 25 cycles instead of 30 for additional fragment and gel purified as above. This fragment was then radioactively

labeled with ^{32}P-dCTP using Rediprime II™ (Amersham), a random prime labeling system, according to the manufacturer's specifications. The probe was then purified using a Nuc-Trap™ column (Stratagene) according to the manufacturer's instructions. ExpressHyb™ (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 65°C using 1.05 x 10^6 cpm/ml of labeled probe. The blots were then washed 4 times for 15 minutes in 2X SSC/1% SDS at 25°C, followed by two 30 minute washes in 0.1X SSC/0.1% SDS at 55°C. Transcripts of approximately 3 kb were detected in heart, brain, placenta, lung , liver, kidney, pancreas, spleen, prostate, testis, ovary, small intestine, colon, stomach, thyroid, spinal cord, lymph node, trachea and adrenal gland. The highest expression was seen in ovary and testis, moderate levels of expression were seen in heart, placenta, lung, liver, kidney, prostate and pancreas; and lower levels of expression were seen in adult brain, spleen, small intestine and colon and thyroid tissue, and the other tissues listed above.

Additional analysis was carried out on a Human Normal Brain Blot I (Invitrogen, Carlsbad, CA) using the probe and hybridization conditions described above. A faint approximately 3 kb transcript was seen in all tissue sections indicating that ZSLIT3 mRNA is expressed in adult brain tissues. ZSLIT3 is expected to show expression in human fetal brain blots as well.

Dot Blots were also performed using Human RNA Master Blots™ (Clontech). The methods and conditions for the Dot Blots are the same as for the Multiple Tissue Blots described above. Dot blot had strongest signals in aorta, liver, kidney, small intestine, lung, placenta, fetal kidney and fetal spleen. The dot blot fetal tissue signals were strongest in heart, kidney, liver, spleen, and lung, and weaker in brain and thymus.

Example 3

Using PCR to Assess ZSLIT3 Expression in Tissues and Cell Lines

A PCR cDNA panel screen was performed on ZSLIT3 using Marathon cDNAs generated in house using the Marathon cDNA Amplification Kit (Clontech). The panel included the following tissues and cell lines: Daudi, HuH7, adrenal gland,

bladder, bone marrow, brain, CaCO2, CD4+, CD8+, cervix, fetal brain, fetal heart, fetal kidney, fetal lung, fetal muscle, fetal skin, glioblastoma, heart, liver, HOS, K562, kidney, lung, lymph node, melanoma, MLR, MG63, pancreas, pituitary gland, placenta, prostate, rectum, SAOS2, salivary gland, skeletal muscle, small intestine, spinal cord, spleen, stomach, T47D, testis , thymus, thyroid, trachea, U2OS and uterus. The PCR reactions were performed with primers ZC23,641 (SEQ ID NO:5) and ZC23,642 (SEQ ID NO:6) using the same conditions as described in Example 2. CaCO2 (intestinal carcinoma), glioblastoma, SAOS2 (primary osteogenic sarcoma), MG63 (osteogenic sarcoma) and T47D (breast carcinoma) reactions gave a signal, as evinced by the correct 116 bp product.

Example 4

Chromosomal Assignment and Placement of ZSLIT3

ZSLIT3 is mapped to a human chromosome, such as chromosome 16, using the commercially available GeneBridge 4 Radiation Hybrid Panel (Research Genetics, Inc., Huntsville, AL). The GeneBridge 4 Radiation Hybrid Panel contains DNAs from each of 93 radiation hybrid clones, plus two control DNAs (the HFL donor and the A23 recipient). A publicly available WWW server (http://www-genome.wi.mit.edu/cgi-bin/contig/ rhmapper.pl) allows mapping relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map of the human genome (the "WICGR" radiation hybrid map) which was constructed with the GeneBridge 4 Radiation Hybrid Panel.

For the mapping of ZSLIT3 with the GeneBridge 4 RH Panel, 20 µl reactions are set up in a 96-well microtiter plate (Stratagene, La Jolla, CA) and used in a RoboCycler Gradient 96 thermal cycler (Stratagene). Each of the 95 PCR reactions consist of 2 µl 10X KlenTaq PCR reaction buffer (Clontech), 1.6 µl dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1 µl sense primer, 1 µl antisense primer, 2 µl *Redi*Load (Research Genetics, Inc.), 0.4 µl 50X Advantage KlenTaq Polymerase Mix (Clontech), 25 ng of DNA from an individual hybrid clone or control and ddH_2O for a total volume of 20 µl. The reactions are overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions are, for example, as follows: an

initial 1 cycle 5 minute denaturation at 95°C, 35 cycles of a 1 minute denaturation at 95°C, 1 minute annealing at 66°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions are separated by electrophoresis on a 2% agarose gel (Life Technologies, Gaithersburg, MD).

An STS is defined by a pair of oligonucleotide primers that are used in a polymerase chain reaction, as describe above, to specifically detect this site in the presence of all other genomic sequences. Since STSs are based solely on DNA sequence they can be completely described within an electronic database, for example, Database of Sequence Tagged Sites (dbSTS), GenBank, (National Center for Biological Information, National Institutes of Health, Bethesda, MD http://www.ncbi.nlm.nih.gov), and can be searched with a gene sequence of interest for the mapping data contained within these short genomic landmark STS sequences, or on the WICGR radiation hybrid map. Proximal and distal framework markers can be determined as well. The use of surrounding markers will position ZSLIT3 in a defined region on the integrated LDB chromosome map (The Genetic Location Database, University of Southhampton, WWW server: http://cedar. genetics.soton.ac.uk/public_html/).

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

What is claimed is:

1. An isolated polynucleotide that encodes a slit protein polypeptide comprising a sequence of amino acid residues that is at least 90% identical to an amino acid sequence from the group of:

(a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe);

(b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and

(c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile).

2. An isolated polynucleotide according to claim 1, wherein the polynucleotide is from the group of:

(a) a polynucleotide sequence as shown in SEQ ID NO:1 from nucleotide 157 to nucleotide 861;

(b) a polynucleotide sequence as shown in SEQ ID NO:1 from nucleotide 70 to nucleotide 2019;

(c) a polynucleotide sequence as shown in SEQ ID NO:1 from nucleotide 1 to nucleotide 2019; and

(d) a polynucleotide sequence complementary to (a), (b), or (c).

3. An isolated polynucleotide sequence according to claim 1, wherein the polynucleotide comprises nucleotide 1 to nucleotide 2019 of SEQ ID NO:3.

4. An isolated polynucleotide according to claim 1, wherein the slit protein polypeptide comprises a sequence of amino acid residues from the group of:

(a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe);

(b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and

(c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile).

5. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA segment encoding a slit protein polypeptide as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and

a transcription terminator,

wherein the promoter is operably linked to the DNA segment, and the DNA segment is operably linked to the transcription terminator.

6. An expression vector according to claim 5, further comprising a secretory signal sequence operably linked to the DNA segment.

7. A cultured cell comprising an expression vector according to claim 5, wherein the cell expresses a polypeptide encoded by the DNA segment.

8. A DNA construct encoding a fusion protein, the DNA construct comprising:

a first DNA segment encoding a polypeptide comprising a sequence of amino acid residues from the group of:

(a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 23 (Gly);

(b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 52 (Pro);

(c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe);

(d) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 288 (Pro), to amino acid number 408 (Asp);

(e) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 298 (Asn), to amino acid number 350 (Pro);

(f) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 409 (Cys), to amino acid number 441 (Cys);

(g) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 442 (Glu), to amino acid number 673 (Ile);

(h) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and

at least one other DNA segment encoding an additional polypeptide,

wherein the first and other DNA segments are connected in-frame; and

wherein the first and other DNA segments encode the fusion protein.

9. An expression vector comprising the following operably linked elements:

a transcription promoter;

a DNA construct encoding a fusion protein according to claim 8; and

a transcription terminator,

wherein the promoter is operably linked to the DNA construct, and the DNA construct is operably linked to the transcription terminator.

10. A cultured cell comprising an expression vector according to claim 9, wherein the cell expresses a polypeptide encoded by the DNA construct.

11. A method of producing a fusion protein comprising:

culturing a cell according to claim 10; and

isolating the polypeptide produced by the cell.

12. An isolated slit protein polypeptide comprising a sequence of amino acid residues that is at least 90% identical to an amino acid sequence from the group of:

(a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe);

(b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and

(c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile).

13. An isolated polypeptide according to claim 12, wherein the polypeptide comprises a sequence of amino acid residues from the group of:

(a) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 53 (Asp), to amino acid number 287 (Phe);

(b) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 673 (Ile); and

(c) the amino acid sequence as shown in SEQ ID NO:2 from amino acid number 1 (Met), to amino acid number 673 (Ile).

14. A method of producing a slit protein polypeptide comprising:

culturing a cell according to claim 7; and

isolating the slit protein polypeptide produced by the cell.

15. A method of producing an antibody to a polypeptide comprising:

inoculating an animal with a polypeptide from the group of:

(a) a polypeptide consisting of 30 to 649 amino acids, wherein the polypeptide is identical to a contiguous sequence of amino acids in SEQ ID NO:2 from amino acid number 24 (Cys) to amino acid number 673 (Ile);

(b) a polypeptide according to claim 13;

(c) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 24 (Cys), to amino acid number 52 (Pro);

(d) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 288 (Pro), to amino acid number 408 (Asp);

(e) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 298 (Asn), to amino acid number 350 (Pro);

(f) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 409 (Cys), to amino acid number 441 (Cys);

(g) a polypeptide consisting of the amino acid sequence of SEQ ID NO:2 from amino acid number 442 (Glu), to amino acid number 673 (Ile); and

wherein the polypeptide elicits an immune response in the animal to produce the antibody; and

isolating the antibody from the animal.

16. An antibody produced by the method of claim 15, which binds to a polypeptide of SEQ ID NO:2.

17. The antibody of claim 16, wherein the antibody is a monoclonal antibody.

18. An antibody that specifically binds to a polypeptide of claim 12 or 13.

19. A method of detecting, in a test sample, the presence of a modulator of ZSLIT3 protein activity, comprising:

transfecting a ZSLIT3-responsive cell, with a reporter gene construct that is responsive to a ZSLIT3-stimulated cellular pathway; and

producing a ZSLIT3 polypeptide by the method of claim 14; and

adding the ZSLIT3 polypeptide to the cell, in the presence and absence of a test sample; and

comparing levels of response to the ZSLIT3 polypeptide, in the presence and absence of the test sample, by a biological or biochemical assay; and

determining from the comparison, the presence of the modulator of ZSLIT3 activity in the test sample.

20. A method for detecting a genetic abnormality in a patient, comprising:

obtaining a genetic sample from a patient;

producing a first reaction product by incubating the genetic sample with a polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1, under conditions wherein said polynucleotide will hybridize to complementary polynucleotide sequence;

visualizing the first reaction product; and

comparing said first reaction product to a control reaction product from a wild type patient, wherein a difference between said first reaction product and said control reaction product is indicative of a genetic abnormality in the patient.

21. A method for detecting a cancer in a patient, comprising:

obtaining a tissue or biological sample from a patient;

incubating the tissue or biological sample with an antibody of claim 18 under conditions wherein the antibody binds to its complementary polypeptide in the tissue or biological sample;

visualizing the antibody bound in the tissue or biological sample; and

comparing levels of antibody bound in the tissue or biological sample from the patient to a normal control tissue or biological sample,

wherein an increase or decrease in the level of antibody bound to the patient tissue or biological sample relative to the normal control tissue or biological sample is indicative of a cancer in the patient.

22. A method for detecting a cancer in a patient, comprising:

obtaining a tissue or biological sample from a patient;

labeling a polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO:1 or the complement of SEQ ID NO:1;

incubating the tissue or biological sample with under conditions wherein the polynucleotide will hybridize to complementary polynucleotide sequence;

visualizing the labeled polynucleotide in the tissue or biological sample; and

comparing the level of labeled polynucleotide hybridization in the tissue or biological sample from the patient to a normal control tissue or biological sample,

wherein an increase or decrease in the labeled polynucleotide hybridization to the patient tissue or biological sample relative to the normal control tissue or biological sample is indicative of a cancer in the patient.

SEQUENCE LISTING

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Leu Gly Pro Gly Val Gln Gly Cys Pro Ser Gly Cys Gln Cys Ser Gln
            20                  25                  30

cca cag aca gtc ttc tgc act gcc cgc cag ggg acc acg gtg ccc cga      144
Pro Gln Thr Val Phe Cys Thr Ala Arg Gln Gly Thr Thr Val Pro Arg
        35                  40                  45

gac gtg cca ccc gac acg gtg ggg ctg tac gtc ttt gag aac ggc atc      192
Asp Val Pro Pro Asp Thr Val Gly Leu Tyr Val Phe Glu Asn Gly Ile
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acc atg ctc gac gca ggc agc ttt gcc ggc ctg ccg ggc ctg cag ctc      240
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        115                 120                 125

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Leu Tyr Leu Gly Lys Asn Arg Ile Arg His Ile Gln Pro Gly Ala Phe
    130                 135                 140

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145                 150                 155                 160

cgg gca ctg ccc ccg ctg cgc ctg ccc cgc ctg ctg ctg ctg gac ctc      528
Arg Ala Leu Pro Pro Leu Arg Leu Pro Arg Leu Leu Leu Leu Asp Leu
                165                 170                 175

agc cac aac agc ctc ctg gcc ctg gag ccc ggc atc ctg gac act gcc      576
Ser His Asn Ser Leu Leu Ala Leu Glu Pro Gly Ile Leu Asp Thr Ala
            180                 185                 190

aac gtg gag gcg ctg cgg ctg gct ggt ctg ggg ctg cag cag ctg gac      624
Asn Val Glu Ala Leu Arg Leu Ala Gly Leu Gly Leu Gln Gln Leu Asp
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Pro Glu Asp Leu Ala Gly Leu Ala Ala Leu Gln Glu Leu Asp Val Ser
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cgg ctg ctc ctg gag ctt gac tac gcc gac ttt ggc tgc cca gcc acc     1056
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ccc gaa ggc ttc acg ggc ctg tac tgt gag agc cag atg ggg cag ggg     1344
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aca cgg ccc agc cct aca cca gtc acg ccg agg cca cca cgg tcc ctg     1392
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acc tat cgc aac cta tcg ggc cct gat aag cgg ctg gtg acg ctg cga     1536
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            500                 505                 510

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gag gga gtg aag gtc ccc ttg gag cca ggc ccg aag gca aca gag ggc     1920
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625                 630                 635                 640

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225                 230                 235                 240
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305                 310                 315                 320
Ala Ser Pro Glu Glu Thr Arg Cys His Phe Pro Pro Lys Asn Ala Gly
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Arg Leu Leu Leu Glu Leu Asp Tyr Ala Asp Phe Gly Cys Pro Ala Thr
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Thr Thr Thr Ala Thr Val Pro Thr Thr Arg Pro Val Val Arg Glu Pro
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Ala Thr Tyr Ser Val Cys Val Met Pro Leu Gly Pro Gly Arg Val Pro
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/34230

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/12 C12N5/10 C07K14/47 C07K16/18 G01N33/50
G01N33/566 C12Q1/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, BIOSIS, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE EMBL SEQUENCES 'Online! Accession No. AC005222, 2 July 1998 (1998-07-02) RICKE D.O.: "Homo sapiens chromosome 16, cosmid clone RT163 (LANL)" XP002162951 seq. ID 1 corresponds to nt 22260-24281	1,3,4, 12,13
A	WO 99 23219 A (OSIRIS THERAPEUTICS INC) 14 May 1999 (1999-05-14) the whole document	1-22
A	JP 10 087699 A (ASAHI CHEM IND CO LTD) 7 April 1998 (1998-04-07) the whole document	1-22

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 March 2001	26/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Galli, I

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INTERNATIONAL SEARCH REPORT

Information on patent family members

h ional Application No
PCT/US 00/34230

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9923219 A	14-05-1999	AU 1203999 A	24-05-1999
JP 10087699 A	07-04-1998	NONE	